HYDROGEN / DEUTERIUM FRACTIONATION FACTORS OF THE AQUEOUS LIGAND OF COBALT IN $Co(H_2O)_6^{2+}$ AND Co(II)-SUBSTITUTED CARBONIC ANHYDRASE

By

JAMES WEB KASSEBAUM

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1988

ACKNOWLEDGEMENTS

I wish to thank Dr. David Silverman for his thoughtfulness and guidance shown to me during the work presented in this dissertation. I have benefitted greatly from his scientific insight into experimental design and his careful attention to excellence in scientific writing. I also wish to thank very much Dr. C. K. Tu and Mr. George Wynns for their help in experimental procedures and for many helpful discussions. I acknowledge the very valuable advice of the members of my committee, Dr. E. Raymond Andrew, Dr. Robert Cohen, Dr. Russell Drago, and Dr. Daniel Purich. Finally, I especially thank Dr. Seymour Koenig at the IBM T. J. Watson Research Center in Yorktown Heights, New York, for allowing me to visit his laboratory and use the field-cycling relaxometer which added greatly to the scope of this work.

TABLE OF CONTENTS

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

HYDROGEN / DEUTERIUM FRACTIONATION FACTORS OF
THE AQUEOUS LIGAND OF COBALT IN $Co(H_2O)_6^{2+}$ AND
Co(II)-SUBSTITUTED CARBONIC ANHYDRASE

By

James Web Kassebaum

December, 1988

Chairman: Dr. David N. Silverman
Major Department: Biochemistry and Molecular Biology

I have measured the hydrogen / deuterium fractionation factor for the rapidly exchanging aqueous ligands of cobalt in $Co(H_2O)_6^{2+}$ and in three Co(II)-substituted isozymes of carbonic anhydrase. The fractionation factor was determined from NMR relaxation rates at 300 MHz of the protons of water in mixed solutions of H_2O and D_2O containing these complexes. In each case, the paramagnetic contribution to $1/T_2$ was greater than to $1/T_1$, consistent with a chemical shift mechanism affecting $1/T_2$. The fractionation factors obtained from T_1 for $Co(H_2O)_6^{2+}$ and for the isozymes of Co(II)-substituted carbonic anhydrase were close to the fractionation factor for bulk water, which is unity. The fractionation factors obtained from T_2 were 0.73 ± 0.02 for $Co(H_2O)_6^{2+}$, 0.72 ± 0.02 for Co(II)-substituted carbonic anhydrase I, 0.77 ± 0.01 for Co(II)-substituted carbonic anhydrase II, and 1.00 ± 0.07 for Co(II)-substituted carbonic anhydrase III. I concluded that fractionation factors in these cases determined from T_1 and T_2 measured isotope preferences for different populations of ligand sites. I suggest that since T_2 has a large contribution from

a chemical shift mechanism, the fractionation factor determined from T_2 has a large contribution of the fractionation of inner shell ligands. The fractionation factors determined from T_1 are close to unity, the value of the fractionation factor of bulk water, and contain a larger contribution of the fractionation of outer shell water.

The fractionation factor of $Co(H_2O)_6^{2+}$ was used to interpret the solvent hydrogen isotope effects on the formation of complexes of cobalt with the bidentate ligands glycine, *N,N*-dimethylglycine, and acetylacetone. The contribution of the fractionation factor of the inner shell water in $Co(H_2O)_6^{2+}$ did not account completely for the measured isotope effect, and I suggest that the hydrogen / deuterium fractionation of outer shell water makes a large contribution to the isotope effect on the formation of these complexes.

The solvent hydrogen isotope effect on the equilibrium binding of iodide ion to Co(II)-substituted carbonic anhydrase I and II was determined and then interpreted using the fractionation factor of the aqueous ligand of cobalt at the active site. This fractionation factor could not account for the measured isotope effect, implying that the hydrogen / deuterium fractionation of additional groups, or water associated with the enzyme, changes on going from the reactant state to the product state when iodide binds to Co(II)-substituted carbonic anhydrase I and II. Although not yet helpful in interpreting the complex contributions to the isotope effects in the enzymatic catalysis, these hydrogen / deuterium fractionation factors for the water bound to cobalt in carbonic anhydrase can be significantly different from the fractionation factor for solvent water and may be sensitive to the active site environment in these homologous isozymes of carbonic anhydrase.

CHAPTER 1
INTRODUCTION

The effect of an isotopic substitution on rate and equilibrium constants associated with a chemical reaction can give information about the mechanism of the reaction. One method in making an isotopic substitution is the replacement of D_2O for H_2O as the solvent, which can affect a reaction in two ways. First if water is a substrate in the reaction, then the rate and equilibrium constants can be affected by the presence of D_2O. Also, if there are exchangeable hydrogenic sites in the reactants or products, or, in the case of an enzyme-catalyzed reaction, if there are exchangeable hydrogenic sites on the enzyme, then the rate and equilibrium constants can be affected when D_2O is the solvent, because deuterium will be present in the exchangeable positions.

The difference in a reaction in H_2O and D_2O results from differences in the zero point energy of bonds to H or D (Schowen, 1978). In the case of a reaction rate, if the zero point energy difference for a bond to H and to D decreases on going to the transition state, then the activation energy is greater in the case of D, and the rate is lower. This is an example of a normal isotope effect, in which the rate constant when the bond is to H, k_H, is greater than the rate constant when the bond is to D, k_D. There also exist reactions where $k_D > k_H$, which is known as an inverse isotope effect.

The difference in zero point energy can be expressed in terms of an equilibrium constant for isotope exchange for a particular hydrogenic site. For a single solute species in an isotopically-mixed aqueous solvent, the hydrogen /

deuterium fractionation factor, ϕ, is the equilibrium constant for this isotope exchange reaction

$$R(H) + (HOD) \rightarrow R(D) + (HOH)$$

$$\phi = \frac{[R(D)] / [R(H)]}{[HOD] / [HOH]}$$

in which a solute hydrogenic species can exchange with a solvent hydrogenic species. The fractionation factor measures the tendency of D to accumulate in the solute relative to the deuterium content of bulk solvent. A fractionation factor greater than unity implies that D will accumulate in the solute position relative to the solvent, and that the bond to hydrogen is stronger in the solute position than that bond in the solvent. For a fractionation factor less than unity, the light isotope of hydrogen will accumulate in the solute position, and the bond to hydrogen is weaker in the solute relative to the solvent.

An isotope effect is a function of the fractionation factors of each hydrogenic site in a reaction. For an isotope effect on a kinetic constant,

$$\frac{k_{H_2O}}{k_{D_2O}} = \frac{\prod_{i}^{\text{all reactant state sites}} \phi_i^R}{\prod_{i}^{\text{all transition state sites}} \phi_i^T} \qquad (1\text{-}1)$$

and for an isotope effect on an equilibrium constant,

$$\frac{K_{H_2O}}{K_{D_2O}} = \frac{\prod_{i}^{\text{all reactant state sites}} \phi_i^R}{\prod_{i}^{\text{all product state sites}} \phi_i^P} \qquad (1\text{-}2)$$

where ϕ^R, ϕ^T, and ϕ^P are reactant, transition, and product state hydrogenic site fractionation factors, respectively (Schowen, 1978). Thus it is clear that to understand completely an isotope effect associated with a reaction, the knowledge of the underlying fractionation factors is required.

Values of the fractionation factor for various functional groups are known relative to water as the solvent (Schowen and Schowen, 1982; Kresge *et al.* 1987), and range from 0.4 for some examples of hydrogen bound to sulfur, to 1.5 for one case of a hydrogen bound to a tertiary amine. The majority of these fractionation factors where measured by one of two methods. One involves using NMR chemical shifts to measure the fractionation factor of a hydrogenic site directly. The technique is based on the rapid exchange of hydrogens between solute and solvent species, so that the observed proton resonance is a weighted average of the chemical shift of the solute and solvent positions. The fractionation factor is determined by measuring the chemical shift at different concentrations of solute in solvents of low and high atom fraction of deuterium. Using this method, Gold and Lowe (1968) determined the fractionation factor of the exchangeable hydrogen of acetic acid to be 0.96 ± 0.02. A variation of this method when there is slow exchange of hydrogens between solute and solvent involves measuring the NMR signal areas in mixed isotopic solvents. In this manner Chiang *et al.* (1980) have determined the fractionation factor of the exchanging position of 1,8-Bis(dimethylamino)-naphthalene to be 0.90 ± 0.01, and Szawelski *et al.* (1982) have measured the fractionation factor of the

hydrogen bound to sulfur in mercaptoethanol to be 0.55 ± 0.02. The second method to determine fractionation factors is to measure the equilibrium constant for a reaction as a function of the atom fraction of deuterium in the solvent, and then using relationships like those that lead to equations 1-1 and 1-2 to calculate the fractionation factor from a fit to the data. This method is then indirect and is unable to separate the contribution from the fractionation at one site from contributions from the medium, which may be a factor. Lowe and Smith (1975) have used this method to determine the fractionation factor of the acidic hydrogen in benzoic acid to be 1.02 ± 0.01. Both of these methods for determining fractionation factors lead to precise values for the simple cases cited above involving one exchangeable hydrogen.

The unifying theme of this work is the study of the hydrogen / deuterium fractionation factor properties of the hydrogenic positions of aqueous ligands of metal ions in simple inorganic complexes and carbonic anhydrase. There is a relative lack of information about the fractionation of the hydrogenic sites of the aqueous ligands of metals compared to that of other functional groups. Using an NMR relaxation method, I have measured the fractionation factor of the aqueous ligand of cobalt in some simple inorganic complexes and in Co(II)-substituted carbonic anhydrase. The goal is to use these fractionation factors in the interpretation of isotope effects on the formation of cobalt complexes and on equilibrium and kinetic constants associated with carbonic anhydrase. The fractionation factor of the aqueous ligand of cobalt in these systems represents the individual contributions of those hydrogenic positions to the overall isotope effect on the reactions.

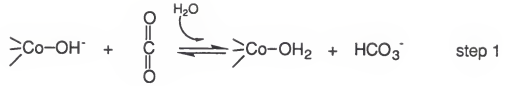
Solvent hydrogen isotope effects in inorganic reactions are relatively little studied compared to those effects in organic reactions (Kresge *et al.* 1987). The data for ligand substitution reactions with transition metal complexes consists of

a small number of solvent hydrogen isotope effects measured for equilibrium constants and for rate constants of reactions with Cr^{3+} and Co^{3+} (Laughton and Robertson, 1969; Gold and Wood, 1982). Kresge *et al.* (1987) have suggested that large rate and equilibrium differences between reactions of transition metal aquacomplexes in H_2O and D_2O should be expected because the hydrogen / deuterium fractionation factor of the water molecules in the inner coordination shell would be raised to the twelfth power for a hexaaquacomplex.

Solvent hydrogen isotope effects on reactions catalyzed by carbonic anhydrase are known and have been used to deduce aspects of the catalytic mechanism (Silverman and Vincent, 1983). Carbonic anhydrase is a zinc metalloenzyme which catalyzes the reversible hydration of carbon dioxide to produce bicarbonate and a proton.

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$$

The zinc can be removed and replaced with cobalt to produce an enzyme with very similar catalytic properties (Lindskog, 1983) and the cobalt, because of its unpaired electrons, provides a spectroscopic probe of the enzyme (Bertini and Luchinat, 1983). In particular, the paramagnetic cobalt will enhance the NMR relaxation rate of water protons in solutions of Co(II)-substituted carbonic anhydrase. The metal ion is at the base of the active site cleft and is coordinated by three histidine ligands with a water molecule as a fourth ligand which can ionize to a hydroxide. The mechanism of CO_2 hydration (scheme 1-1) takes place by direct nucleophilic attack of a metal-bound hydroxide on CO_2 to produce HCO_3^- (Steiner *et al.* 1975), with a water molecule then displacing HCO_3^- to produce a metal-bound water. A proton must then be transferred out of the active site to regenerate the metal-bound hydroxide for the next round of catalysis.



Scheme 1-1: The metal hydroxide mechanism of carbonic anhydrase.

When this reaction is performed in D_2O some very interesting isotope effects are observed. The isotope effect on the steady-state parameter k_{cat} for both native and Co(II)-substituted carbonic anhydrase II is large, 3.8 in the case of native zinc-carbonic anhydrase II (Steiner *et al.* 1975), and 1.8 for Co(II)-substituted carbonic anhydrase II (R. S. Rowlett, unpublished), which means that a primary proton transfer is at least part of the rate limiting step measured by k_{cat}. The ratio k_{cat}/K_m contains steps up to and including the first irreversible step in the reaction, and for the catalyzed reaction the first irreversible step is the release of HCO_3^- from the enzyme. The isotope effect is unity on k_{cat}/K_m for both native zinc-carbonic anhydrase II (Steiner *et al.* 1975) and for Co(II)-substituted carbonic anhydrase II (R. S. Rowlett, unpublished), which implies that the rate determining step measured by k_{cat} is separate and distinct from the interconversion of CO_2 and HCO_3^-. Thus the rate-limiting step measured by k_{cat} is the rate of transfer of a proton from the metal-bound water out of the active site to regenerate the metal-bound hydroxide (step 2 of Scheme 1-1). From this description it is clear that the aqueous ligand of the metal at the active site of carbonic anhydrase is intimately involved in the mechanism of catalysis, and to interpret the isotope effects knowledge of the fractionation factor of the aqueous ligand of the metal is required.

The fractionation factor of the aqueous ligand of a paramagnetic metal can be determined by taking advantage of the paramagnetic contribution to the NMR relaxation rates of water protons in solutions of the metal in which the protons are in fast exchange between the coordination shell of the metal and the bulk solvent. The NMR measurements in this work were performed at a proton resonance frequency of 300 MHz, which is a higher frequency than has been used to study the water proton relaxation enhancement of first row transition metals (Dwek, 1975; Bertini and Luchinat, 1986). Thus the relaxation

mechanisms operating in these systems at 300 MHz need to be understood before the relaxation can be used to measure a fractionation factor, and this is discussed in Chapter 2. Chapter 3 includes the determination of the fractionation factor of $Co(H_2O)_6^{2+}$ and $Ni(H_2O)_6^{2+}$ along with measurements of the solvent hydrogen isotope effect on the formation constants of some cobalt complexes to see if the fractionation factor of $Co(H_2O)_6^{2+}$ can account for any of these effects. In Chapter 4 I have measured the fractionation factor of three mammalian isozymes of Co(II)-substituted carbonic anhydrase using NMR relaxation, and in Chapter 5 I discuss the use of these fractionation factors to interpret solvent hydrogen isotope effects on the equilibrium binding of an inhibitor to carbonic anhydrase and on the catalysis. Finally, the relaxation properties of Co(II)-substituted carbonic anhydrase III have not previously been reported, and in Chapter 6 I discuss experiments to determine the magnetic field and pH dependence of the water proton relaxation enhancement in solutions of Co(II)-substituted carbonic anhydrase III.

CHAPTER 2
WATER PROTON NMR RELAXATION ENHANCEMENT IN SOLUTIONS OF $Co(H_2O)_6^{2+}$ AND $Ni(H_2O)_6^{2+}$

Introduction

The initial studies of the influence of paramagnetic ions on the NMR relaxation rates of water protons were performed at proton resonance frequencies up to 60 MHz (Bernheim *et al.*, 1959; Morgan and Nolle, 1959). These results were interpreted in terms of the Solomon-Bloembergen equations, which describe the relaxation as the sum of an electron-nuclear dipole-dipole interaction and a scalar interaction (Solomon, 1955; Bloembergen, 1957). At a frequency of 20 MHz the water protons in solutions of $Co(H_2O)_6^{2+}$ were observed by Bernheim *et al.* (1959) to relax such that $T_1 \approx T_2$, and this relaxation was also observed at 10 MHz for solutions of $Ni(H_2O)_6^{2+}$ (Morgan and Nolle, 1959). This result is consistent with the electron-nuclear dipole-dipole mechanism dominating the relaxation of the water protons, the correlation time for the interaction being the very short electron spin relaxation time. This short relaxation time for the electron in $Co(H_2O)_6^{2+}$ and $Ni(H_2O)_6^{2+}$ precludes any contribution to the relaxation from the scalar mechanism. In the absence of a scalar contribution, 7/6 would be the largest observable ratio for T_1/T_2.

I have made measurements of the relaxation of water protons in solutions of $Co(H_2O)_6^{2+}$ and $Ni(H_2O)_6^{2+}$ at a proton resonance frequency of 300 MHz. In each case it was observed that the ratio T_1/T_2 was much larger than unity, consistent with a chemical shift mechanism affecting T_2 at this frequency. Because this mechanism will affect T_2 to the greatest extent when the protons

are in the primary hydration shell of the ion, this allowed a qualitative differentiation between the relaxation of inner and outer shell water.

Experimental Procedure

The NMR relaxation times T_1 and T_2 of water protons were measured at a frequency of 300 MHz on a Nicolet NT-300 spectrometer at 23 °C. T_1 was measured by the inversion recovery method according to Freeman *et al.* (1980) and T_2 was measured by the Carr-Purcell-Meiboom-Gill sequence (Meiboom and Gill, 1958). The observed relaxation of water protons in a solution of a paramagnetic ion is the sum of the relaxation due to the paramagnetic ion, $1/T_{ip}$ (i=1,2), and the relaxation in the absence of the ion, $1/T_{io}$,

$$1/T_i = 1/T_{ip} + 1/T_{io} \qquad (2\text{-}1)$$

The contribution of the paramagnetic ion to the longitudinal relaxation is given by (Luz and Meiboom, 1964)

$$1/T_{1p} = p'[1/(T_{1m} + \tau_m)] \qquad (2\text{-}2)$$

and the paramagnetic contribution to the transverse relaxation is given by (Swift and Connick, 1962)

$$1/T_{2p} = p' \left[\frac{(1/T_{2m})(1/T_{2m} + 1/\tau_m) + \Delta\omega_m^2}{\tau_m((1/T_{2m} + 1/\tau_m)^2 + \Delta\omega_m^2)} \right] \qquad (2\text{-}3)$$

where T_{im} is the relaxation time of a proton in the hydration shell of the metal and is described by the Solomon-Bloembergen equations, τ_m is the lifetime of a proton in the hydration shell, and p' is q[M]/55.5 where q is the hydration number and [M] is the concentration of the metal ion. $\Delta\omega_m$ is the chemical shift difference between a proton in the primary hydration shell and a proton in the

free solvent site. If $\Delta\omega_m^2$ is small relative to the other terms, equation 2-3 reduces to equation 2-2. For these metal ions, $1/T_{io}$ was taken as the relaxation of water containing only buffer, with no added metal ions. R_i, the relaxivity, is defined as the paramagnetic relaxation per millimolar concentration of metal ion.

$$R_i = (1/T_{ip})/ ([Metal] \times 1000) \qquad (2\text{-}4)$$

Solutions of $Co(H_2O)_6^{2+}$ and $Ni(H_2O)_6^{2+}$ for NMR measurements contained ultra-pure $CoCl_2$ or $NiSO_4$ (Aldrich Gold-Label) with 5×10^{-4} M acetic acid buffer at pH = 4.4. This pH was necessary to preclude the formation of multi-nuclear hydrolysis products (Baes and Mesmer, 1976). When EDTA was present no acetic acid was used. Water used for solution preparations was distilled and passed through two ion-exchange resin cartridges (Cole-Palmer 1506-35). D_2O (99.8%) was stirred with activated charcoal, the charcoal filtered out, and then the D_2O was distilled.

Results

The paramagnetic contribution to the relaxivity R_2 of water protons in solutions of Co^{2+} and Ni^{2+} at 300 MHz was greater than that obtained at lower frequencies by previous investigators (Table 2-1). For Co^{2+}, R_1 at 300 MHz and 20 MHz were close in value while R_2 was much larger at 300 MHz than at 20 MHz. For Ni^{2+}, R_1 at 300 MHz was slightly larger than at 10 MHz which is understood to be a result of a field dependent increase in the electron relaxation time (Friedman *et al.,* 1979), but R_2 was much larger at 300 MHz than at 10 MHz.

The addition of EDTA to the solutions of $CoCl_2$ and $NiSO_4$ reduced R_1 and R_2 at 300 MHz (Figures 2-1 and 2-2). When an equimolar amount of EDTA

Table 2-1: Paramagnetic contribution to the relaxivities of water protons observed for aqueous solutions of $CoCl_2$ and $NiSO_4$.

	frequency (MHz)	R_1 ($mM^{-1}\,s^{-1}$)	R_2 ($mM^{-1}\,s^{-1}$)	T_{1p}/T_{2p}
$Co(H_2O)_6^{2+}$				
	20 [a]	0.18	0.21	1.2
	300 [b]	0.16	2.0	12.5
$Ni(H_2O)_6^{2+}$				
	10 [c]	0.64	0.71	1.1
	300 [b]	0.95	2.3	2.4

[a] From Bernheim *et al.* (1959) for an unbuffered solution of 0.5 M $CoCl_2$ at 25 °C.

[b] Determined in this work at 23 °C for 10 mM $CoCl_2$ or 10 mM $NiSO_4$ which contained 10% D_2O by volume and 5 x 10^{-4} M acetic acid buffer. The pH was 4.4.

[c] From Morgan and Nolle (1959) for a solution of 10 mM $Ni(NO_3)_2$ at 27 °C in 0.1 M perchloric acid.

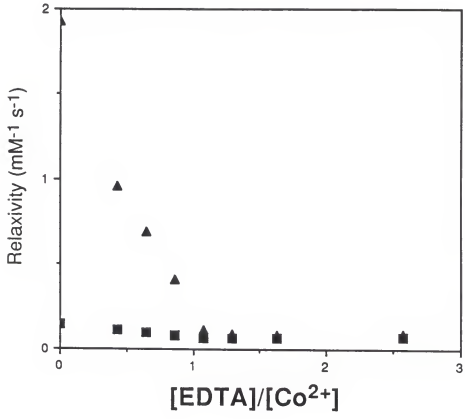


Figure 2-1: The relaxivity of the protons of water at 300 MHz due to Co^{2+} in solutions of 10 mM $CoCl_2$ as a function of the molar ratio of EDTA to Co^{2+} at 23 °C. Solutions contained 50% D_2O by volume. R_1 (■), R_2 (▲).

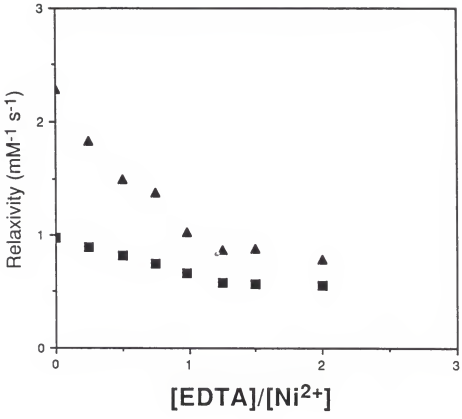


Figure 2-2: The relaxivity of the protons of water at 300 MHz due to Ni^{2+} in solutions of 10 mM $NiSO_4$ as a function of the molar ratio of EDTA to Ni^{2+} at 23 °C. Solutions contained 50% D_2O by volume. R_1 (■), R_2 (▲).

was added to solutions of Co^{2+}, R_1 was reduced to 47% of the value of R_1 in the absence of EDTA, R_2 was reduced to 5% of the value of R_2 in the absence of EDTA, and T_{1p}/T_{2p} was 1.2. The remaining paramagnetic relaxivity due to the CoEDTA complex was $R_1 = 0.075\ mM^{-1}\ s^{-1}$ and $R_2 = 0.091\ mM^{-1}\ s^{-1}$. When an equimolar amount of EDTA was added to solutions of Ni^{2+}, R_1 was reduced to 58% of the value of R_1 in the absence of EDTA, R_2 was reduced to 34% of the value of R_2 in the absence of EDTA, and T_{1p}/T_{2p} was 1.4. The remaining paramagnetic relaxivity due to the NiEDTA complex was $R_1 = 0.55\ mM^{-1}\ s^{-1}$ and $R_2 = 0.78\ mM^{-1}\ s^{-1}$.

The observed change in the chemical shift caused by the paramagnetic metal, $\Delta\omega$, at 300 MHz for a 10 mM solution of $CoCl_2$ was measured to be 158 Hz. The measured change in the chemical shift, $\Delta\omega$, is the difference between the chemical shift of water protons in water containing only buffer and the chemical shift of water protons in a solution of a paramagnetic metal. This was measured with tetramethylsilane as an external reference using coaxial tubes. The influence of this chemical shift on the transverse relaxation of $Co(H_2O)_6^{2+}$ solutions at 20 MHz and 300 MHz was estimated using equation 2-3 (Table 2-2). In the calculations, I used $\tau_m = 7.4 \times 10^{-7}$ s determined by Swift and Connick (1962) using ^{17}O exchange. Also, I used $T_{1m} = T_{2m}$ as predicted by the Solomon-Bloembergen equations for $Co(H_2O)_6^{2+}$ at 20 MHz and 300 MHz. From the observed T_1 at 300 MHz, T_{1m} was calculated (from equations 2-1 and 2-2) to be 6.62×10^{-4} s. From the data of Bernheim *et al.* (1959) $T_{1m} = 5.95 \times 10^{-4}$ s at 20 MHz. Since $T_{1m} >> \tau_m$, the fast exchange limit applies at 20 MHz and at 300 MHz. $\Delta\omega_m$ was calculated from $\Delta\omega$ at 300 MHz using $\Delta\omega = p' \Delta\omega_m$ which is valid in the fast exchange limit (Swift and Connick, 1962). I determined $\Delta\omega_m$ to be 1.46×10^5 Hz at 300 MHz. Since $\Delta\omega_m$ is directly proportional to the precessional frequency, $\Delta\omega_m$ at 20 MHz is 9.73×10^3 Hz. The results using

these values with equation 2-3 are in Table 2-2 and are in good agreement with the experimental results in Table 2-1.

Discussion

The results at 300 MHz of T_{1p}/T_{2p} much greater than unity for solutions of both $Co(H_2O)_6^{2+}$ and $Ni(H_2O)_6^{2+}$ suggest that a relaxation mechanism different from the dipole-dipole mechanism is present at this frequency. The data in Table 2-1 indicate that this mechanism primarily, if not exclusively, affects the transverse relaxation. I have considered three possible causes of this field-dependent increase in $1/T_{2p}$. The scalar contribution to $1/T_{2m}$ has a correlation time equivalent to the electron relaxation time which is field dependent (Bloembergen and Morgan, 1961). However, I have rejected a contribution from the scalar term because it would require an unreasonably large increase in the electron spin relaxation time at 300 MHz. When the electron relaxation time is less than both the rotational correlation time and τ_m, a Curie spin mechanism can result (Gueron, 1975). This Curie spin is the thermal average of an electron spin which can become large at high field and can cause an increase in T_{1m}/T_{2m}. However, I also reject a contribution from this mechanism because the rotational correlation time for a hexaaquacomplex is too short to allow the Curie spin to become effective.

Melamud and Mildvan (1975) observed a field-dependent increase in $1/T_{2p}$ for water protons in solutions of $Co(H_2O)_6^{2+}$ and concluded that the $\Delta\omega_m^2$ term in equation 2-3, a chemical shift mechanism, made a major contribution at the higher frequencies. I support this conclusion in two ways. First, I have calculated the magnitude of the chemical shift contribution to the transverse relaxation of $Co(H_2O)_6^{2+}$ solutions at 20 MHz and 300 MHz using equation 2-3. These calculations demonstrate that the observed value of T_{1p}/T_{2p} at 300 MHz is consistent with a chemical shift mechanism which affects T_{2p} (compare

Table 2-2: Calculated paramagnetic relaxivities of water protons in solutions containing Co^{2+}.

frequency (MHz)	R_1 ($mM^{-1}\,s^{-1}$)	R_2 ($mM^{-1}\,s^{-1}$)	T_{1p}/T_{2p}
20	0.18	0.19	1.1
300	0.16	1.8	11.2

Note: Calculated using equations 2-2 to 2-4 in the text. The calculations used $\tau_m = 7.4 \times 10^{-7}$ s (Swift and Connick, 1962), the 20 MHz relaxation data from Bernheim *et al.* (1959), and the 300 MHz relaxation data and chemical shift determined in this work. A further description is in the text.

Tables 2-1 and 2-2). The chemical shift does not have an effect at 20 MHz at which frequency $\Delta\omega_m^2 << 1/T_{2m}\tau_m$, $1/T_{2m}^2$. The large chemical shift at 300 MHz due to the addition of Co^{2+} is the result of contact interactions which result from the delocalization of unpaired electron spin density. At lower magnetic field strengths this mechanism has been observed to affect T_2 for ^{17}O and ^{19}F in cobalt complexes (Swift and Connick, 1962; Eisenstadt, 1969). The effects on the relaxation when EDTA is added to solutions of $Co(H_2O)_6^{2+}$ is the second way I support the chemical shift mechanism. The chemical shift mechanism will affect T_{2p} to the greatest extent when the protons are in the primary hydration shell of the ion because it is a contact interaction. EDTA binds to Co^{2+} (and Ni^{2+}) occupying all coordination sites and thus excludes water molecules from the inner shell. Thus the addition of EDTA would prevent the water protons from experiencing the chemical shift mechanism. The ratio T_{1p}/T_{2p} was near unity for water protons at 300 MHz when an equimolar amount of EDTA was added to solutions of Co^{2+}, consistent with the absence of a chemical shift relaxation mechanism and the dominance of a dipole-dipole relaxation mechanism (Figure 2-1).

When EDTA was added to solutions of Co^{2+}, the percent decrease in the relaxivity R_2 at 300 MHz was much greater than R_1 (Figure 2-1). Since EDTA is excluding water molecules from the inner coordination shell, this suggests that R_2 has a much larger contribution from the relaxation of water protons in the inner shell. The residual paramagnetic relaxivity of the cobalt-EDTA complex is most likely due to water protons in an "outer shell" of the complex. This residual relaxivity makes up a much larger part of R_1 than R_2 observed for $Co(H_2O)_6^{2+}$ solutions at 300 MHz. This suggests that the relaxation of outer shell water contributes to a greater extent to R_1 than to R_2 in $Co(H_2O)_6^{2+}$.

As EDTA was added to solutions of Ni^{2+}, R_2 decreased more than R_1 (Figure 2-2), though the decrease is not as large as seen in solutions of Co^{2+}. Also, the residual relaxivity of the NiEDTA complex is much larger than in solutions of the CoEDTA complex, and this residual relaxivity makes up a large part of both R_1 and R_2. These results for Ni^{2+} suggest that the chemical shift mechanism is responsible for the increase in R_2 at 300 MHz, but that R_1 and R_2 each have a large contribution of outer shell water.

CHAPTER 3
HYDROGEN / DEUTERIUM FRACTIONATION FACTOR OF THE AQUEOUS LIGAND IN $Co(H_2O)_6^{2+}$ AND $Ni(H_2O)_6^{2+}$

Introduction

The hydrogen / deuterium fractionation factor, ϕ, of a metal-bound water is the equilibrium constant for this isotope exchange reaction:

$$M(HOH) + (HOD) \longrightarrow M(HOD) + (HOH)$$

$$\phi = \frac{[M(HOD)] / [M(HOH)]}{[HOD] / [HOH]} \qquad (3\text{-}1)$$

This fractionation factor measures the tendency of deuterium to accumulate at the aqueous ligand of the metal relative to the deuterium content of bulk solvent. Fractionation factors are valuable in interpreting the effects of deuterium on kinetic and equilibrium constants because they represent individual contributions to isotope effects measured relative to the common reference of water. The fractionation of hydrogen isotopes in water bound to metal ions has been considered (Schowen and Schowen, 1982); however, there are few reports of the fractionation factor of the aqueous ligands of a metal. Using NMR methods, Melton and Pollack (1969) have measured such a factor for the water ligands of $Cr(H_2O)_6^{2+}$ ($\phi = 1.00 \pm 0.03$). They relied on the properties of a paramagnetic metal to enhance the NMR relaxation rate of protons of water exchanging rapidly between bulk solvent and the hydration shell of the metal. By measuring the proton relaxation rate as a function of the deuterium content of solvent, the fractionation factor of the metal-bound site can be determined.

Using this method at a proton resonance frequency of 300 MHz, I have measured the fractionation factor of the aqueous ligand of cobalt in $Co(H_2O)_6^{2+}$ and of nickel in $Ni(H_2O)_6^{2+}$. In the case of $Co(H_2O)_6^{2+}$ the value of the fractionation factor was dependent upon whether it was determined from T_1 or T_2; however, the corresponding values of ϕ for $Ni(H_2O)_6^{2+}$ were identical when obtained from T_1 or T_2. The analysis of the relaxation discussed in Chapter 2 can be used to interpret the fractionation factors, and allows a qualitative differentiation between the hydrogen / deuterium fractionation of inner and outer shell water in these complexes.

I have then used the knowledge of the fractionation of $Co(H_2O)_6^{2+}$ to study the solvent hydrogen isotope effects on the formation constants of the reactions of glycine, *N,N*-dimethylglycine, and acetylacetone with Co^{2+}. An isotope effect on an equilibrium constant is the product of the reactant state hydrogenic site fractionation factors divided by the product of the product state hydrogenic site fractionation factors (equation 1-2). In a reaction of Co^{2+} with a bidentate ligand L^{-1} represented as

$$Co(H_2O)_6^{2+} + L^{-1} \longrightarrow Co(H_2O)_4L^{+} + 2\,H_2O \qquad (3\text{-}2)$$

the solvent hydrogen isotope effect is a function of the fractionation of the hydrogenic positions of $Co(H_2O)_6^{2+}$ as well as $Co(H_2O)_4L^{+}$ and H_2O, and the fractionation factor of $Co(H_2O)_6^{2+}$ represents the individual contribution of the hydrogenic positions of $Co(H_2O)_6^{2+}$ to the overall isotope effect. Because the solvent hydrogen isotope effects on the formation constants were measured to be near unity in the case of each of the ligands, I determined that the contributions of the fractionation of the hydrogenic positions of the inner shell water in $Co(H_2O)_6^{2+}$ and in the ligand complexes of cobalt can not account completely for the overall isotope effect. I suggest that the fractionation of outer

shell water also makes a large contribution to the isotope effect on these reactions.

Experimental Procedure

NMR Measurements: Measurements at 300 MHz and solutions were the same as in Chapter 2. Some measurements of T_1 of water protons in solutions of Co^{2+} were performed at frequencies between 0.01 and 50 MHz on a field cycling "relaxometer" at the IBM T. J. Watson Research Center, Yorktown Heights, NY (Koenig *et al.*, 1983).

Measurement of the Hydrogen / Deuterium Fractionation Factor: The measurement of the relaxation of water protons in solutions of varying deuterium content and containing paramagnetic metal ions is given by

$$1/T_{ip} = p'[X]/(1 - n + n\phi) \qquad (3\text{-}3)$$

where n is the atom fraction of deuterium in solvent water, ϕ is the hydrogen / deuterium fractionation factor of the water ligands of the metal, and [X] is the term in brackets in equations 2-2 and 2-3 for i = 1 or 2, respectively. This equation is derived considering the fractionation of isotopes that arises when there is a deuterium content in water. In this case the ratio of protons in the hydration shell of the metal to total protons in solution is $p'/(1-n+n\phi)$ (Schowen and Schowen, 1982). Equation 3-3 can be rearranged to

$$p'T_{ip} = [X]^{-1} - n(1-\phi)[X]^{-1} \qquad (3\text{-}4)$$

Thus $p'T_{ip}$ vs n gives a slope of $-(1-\phi)[X]^{-1}$ and an intercept of $[X]^{-1}$, and ϕ = 1 + slope / intercept.

Determination of the isotope effects on formation constants: The formation constants for the reactions of Co^{2+} with a bidentate ligand, L^{-1}, as in equation 3-2 are defined as

$$k_1 = \frac{[Co(H_2O)_4L^+]}{[Co(H_2O)_6^{2+}]\,[L^{-1}]} \qquad (3\text{-}5)$$

with similar expressions for k_2 and k_3. Potentiometric titrations were performed in H_2O and D_2O (99.8%) containing 1 mM $CoCl_2$ and 4 mM glycine, *N,N*-dimethylglycine, or acetylacetone. The values of pH and pD used in these experiments were corrected from pH meter readings. The correction of a pH meter reading in 100% D_2O is pD = meter reading + 0.4 (Glasoe and Long, 1960). The formation constants were determined by a non-linear least-squares fit of the data (RS1, BBN Software, Cambridge, MA) to the equation derived by Carlson *et al.* (1945) describing $\bar{n}$, the ratio of the concentration of metal-bound ligand to total concentration of metal, as a function of the concentration of coordinating ligand, [A].

$$\bar{n} = \frac{k_1[A] + 2k_1k_2[A]^2 + 3k_1k_2k_3[A]^3}{1 + k_1[A] + k_1k_2[A]^2 + k_1k_2k_3[A]^3} \qquad (3\text{-}6)$$

The calculation of $\bar{n}$ and [A] required knowledge of the acid ionization constants of the free ligands, which were measured by potentiometric titrations in H_2O and D_2O (Table 3-1). The negative logarithm of the acid ionization constants, pK, in H_2O were all within 0.07 pK units from the literature values (Smith and Martell, 1975). The solvent hydrogen isotope effects for glycine expressed as ΔpK where $\Delta pK = pK_{D_2O} - pK_{H_2O}$ were within 0.03 ΔpK units from the values obtained previously by Jencks and Salvesen (1971).

Table 3-1: Solvent hydrogen isotope effects on the acid ionization constants of glycine, *N,N*-dimethylglycine, and acetylacetone.

	$(pK_1)H_2O$ [a]	ΔpK_1	$(pK_2)H_2O$ [b]	ΔpK_2
glycine	2.37 ± 0.02	0.40 ± 0.04	9.72 ± 0.03	0.56 ± 0.05
N,N-dimethylglycine	1.96 ± 0.03	0.40 ± 0.04	9.81 ± 0.03	0.55 ± 0.05
acetylacetone	9.03 ± 0.10	0.62 ± 0.17	-	-

Note: Acid ionization constants were determined in H_2O and D_2O by potentiometric titrations at 23°C. Solutions contained 10 mM of either glycine, *N,N*-dimethylglycine, or acetylacetone.

[a] pK = -log K where K is the acid ionization constant. Standard errors resulted from a non-linear least-squares fit of the titration data. $\Delta pK = pK_{D_2O} - pK_{H_2O}$.

These potentiometric titrations were performed in solutions which contained ultra-pure $CoCl_2$ (Aldrich Gold-Label), and glycine and *N,N*-dimethylglycine from Sigma and acetylacetone from Aldrich. Water used for solution preparations was distilled and passed through two ion-exchange resin cartridges (Cole-Palmer 1506-35). D_2O (99.8%) was stirred with activated charcoal, the charcoal filtered out, and then the D_2O was distilled. Glassware was rinsed with a solution of EDTA prior to use.

Results

Fractionation Factors of $Co(H_2O)_6^{2+}$ and $Ni(H_2O)_6^{2+}$: The relaxation of water protons in solutions of 10 mM $CoCl_2$ and 10 mM $NiSO_4$ as a function of the atom fraction of deuterium in solvent water is shown in Figures 3-1 and 3-2. From these data, the hydrogen / deuterium fractionation factor, ϕ, for $Co(H_2O)_6^{2+}$ and $Ni(H_2O)_6^{2+}$ was obtained (Table 3-2). The value of the fractionation factor was independent of the concentration of metal in the range 0.010 M to 0.025 M. The value of ϕ for $Co(H_2O)_6^{2+}$ was dependent on whether it was obtained from T_1 or T_2; however, the corresponding values of ϕ for $Ni(H_2O)_6^{2+}$ were identical when obtained from T_1 or T_2. In a separate experiment, the fractionation factor from T_1 for $Co(H_2O)_6^{2+}$ was determined at frequencies between .01 and 50 MHz on a field cycling "relaxometer" at the IBM T. J. Watson Research Center and found to be virtually field independent (data not shown).

Solvent Hydrogen Isotope Effects on the Formation Constants of Cobalt Complexes: The formation constants for the reactions of Co^{2+} with glycine, *N,N*-dimethylglycine, and acetylacetone were determined in H_2O and D_2O and are reported as the logarithm of the formation constants (Table 3-3). Representative data of a formation curve (Carlson *et al.,* 1945) for Co^{2+} and glycine are in Figure 3-3 where $\bar{n}$ is plotted as a function of the negative logarithm of the

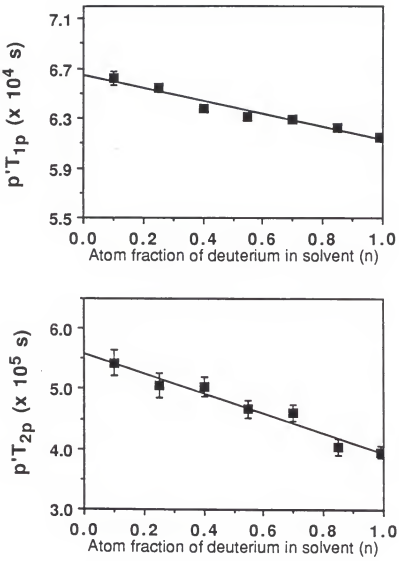


Figure 3-1: The dependence of $p'T_{ip}$ (i = 1,2) at 300 MHz on **n**, the atom fraction of deuterium in solvent water, for a 10 mM solution of $CoCl_2$ at 23 °C. The solutions were buffered at pH 4.4 with 0.5 mM acetic acid. Data are the means and standard errors propagated from NMR measurements.

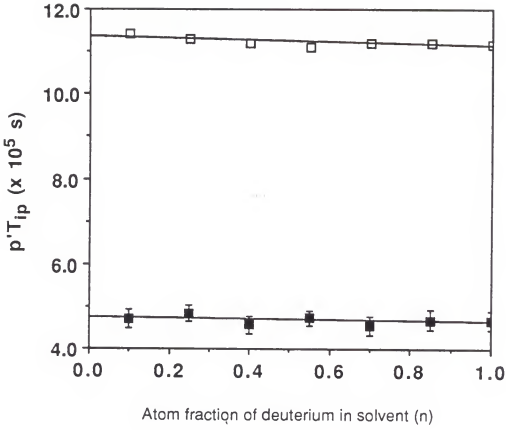


Figure 3-2: The dependence of $p'T_{ip}$ (i = 1,2) at 300 MHz on **n**, the atom fraction of deuterium in solvent water, for a 10 mM solution of $NiSO_4$ at 23 °C. The solutions were buffered at pH 4.4 with 0.5 mM acetic acid. Data are the means and standard errors propagated from NMR measurements. $p'T_{1p}$ (□) $p'T_{2p}$ (■).

Table 3-2: Fractionation factors determined from T_1 and T_2 at 300 MHz for the aqueous ligands of the paramagnetic metal in $Co(H_2O)_6^{2+}$ and $Ni(H_2O)_6^{2+}$.

	ϕ_{T_1}	ϕ_{T_2}
$Co(H_2O)_6^{2+}$	0.95±.01	0.73±.02
$Ni(H_2O)_6^{2+}$	0.99±.01	0.98±.03

Note: Measurements were made at 23 °C for 10 mM $CoCl_2$ or $NiSO_4$ containing 5×10^{-4} M acetic acid buffer at pH 4.4. Data were obtained from the least squares slope and intercept and standard error in a plot such as Fig. 3-1 according to equation 3-4.

Table 3-3: Logarithm of the formation constants determined for the reactions of Co^{2+} with the ligands glycine, *N,N*-dimethylglycine, and acetylacetone in H_2O and D_2O.

		H_2O	D_2O	$(\log k)_{D_2O} - (\log k)_{H_2O}$
glycine	$\log k_1$	5.04 ± 0.04	4.99 ± 0.02	-0.05 ± 0.04
	$\log k_2$	4.32 ± 0.04	4.23 ± 0.02	-0.08 ± 0.05
N,N-dimethylglycine	$\log k_1$	4.14 ± 0.04	4.12 ± 0.06	-0.03 ± 0.07
	$\log k_2$	3.20 ± 0.06	3.18 ± 0.13	-0.01 ± 0.14
acetylacetone	$\log k_1$	5.30 ± 0.07	5.42 ± 0.10	0.12 ± 0.13
	$\log k_2$	4.40 ± 0.09	4.61 ± 0.12	0.21 ± 0.15

Note: Determined by potentiometric titrations of solutions which contained 1 mM $CoCl_2$ and 4 mM of the ligand and fitting the data to equation 3-6. Measurements were made at 23 °C. See equation 3-5 for the definition of k_1.

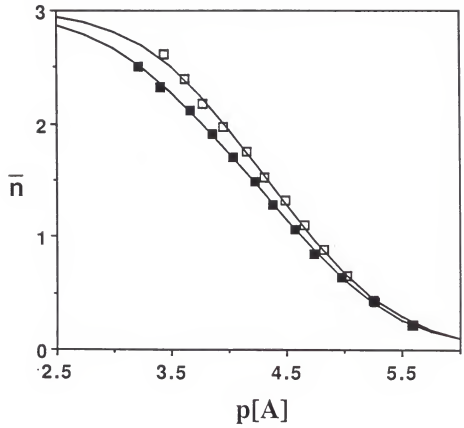


Figure 3-3: The ratio of the concentration of metal-bound ligand to total concentration of metal, $\bar{n}$, as a function of the negative logarithm of the concentration of free glycine in solution existing as NH_2-CH_2-COO^{-1}, p[A] (see equation 3-6). The solutions contained 1 mM total $CoCl_2$ and 4 mM total glycine in H_2O (□) or D_2O (■) and were titrated with NaOH (NaOD). The solid lines are a computer fit to the data using equation 3-6 with the values of the formation constants given in Table 3-3.

concentration of coordinating ligand, p[A]. In the region of the formation curves where p[A] = 3.0 the pH of the solutions was greater than 9.0. Near this pH the hydrolysis of the cobalt-bound water to a cobalt-bound hydroxide occurs in $Co(H_2O)_6^{2+}$ (Baes and Mesmer, 1976) and this same hydrolysis may be occurring in the water ligands of the cobalt complexes with the bidentate ligands. The data in the region p[A] = 3.0 was therefore difficult to interpret, and I was unable to obtain precise values for k_3. For glycine and acetylacetone in H_2O the values of log k_1 were within 0.1 log unit and the values of log k_2 were within 0.3 log unit of the literature values (Smith and Martell, 1975; no literature value was available for *N,N*-dimethylglycine). The solvent hydrogen isotope effects on these formation constants were all unity within experimental uncertainty (Table 3-3), with the possible exception of k_2 for acetylacetone.

For the binding of a ligand L^{-1} to cobalt as in equation 3-2 the solvent hydrogen isotope effect is (see equation 1-2)

$$\frac{(k_1)_{H_2O}}{(k_1)_{D_2O}} = \frac{(\phi Co(H_2O)_6^{2+})^{12}}{(\phi Co(H_2O)_4(L)^{+})^8(\phi H_2O)^4} \qquad (3\text{-}7)$$

and from this the fractionation factor of the remaining metal-bound waters in $Co(H_2O)_4L^+$ can be calculated by using the measured isotope effect, the measured fractionation factor for $Co(H_2O)_6^{2+}$ from NMR relaxation, and the fractionation factor for H_2O which is 1.0 (Schowen and Schowen, 1982). An analogous calculation can be done to determine the fractionation factor of the remaining metal-bound waters in $Co(H_2O)_2(L)_2$. Fractionation factors ϕ_{T_1} and ϕ_{T_2} for $Co(H_2O)_6^{2+}$ have been measured from the NMR relaxation times T_1 and T_2 of water protons (Table 3-2). The results of these calculations from equation

3-7 with glycine, *N,N*-dimethylglycine, and acetylacetone are in Table 3-4 using ϕ_{T_1} and ϕ_{T_2} for $Co(H_2O)_6^{2+}$.

I have also tried to measure the fractionation factor for these coordinated water molecules in the complexes directly, using the NMR relaxation method used to determine the fractionation factor of $Co(H_2O)_6^{2+}$. However, these solutions of Co^{2+} and a bidentate ligand L^- consist of an equilibrium mixture of $Co(H_2O)_6^{2+}$, $Co(H_2O)_4L^+$, $Co(H_2O)_2L_2$, and CoL_3^- and the observed paramagnetic relaxation is the sum of the relaxation of each of these four species. This presents difficulties in obtaining the fractionation factor of one species from these measurements.

Discussion

The definition of the hydrogen / deuterium fractionation factor I have given (equation 3-1) assumes the rule of the geometric mean to be valid (Schowen and Schowen, 1982); that is, the fractionation factor of one particular hydrogenic site is independent of the isotopic composition of any other site. I will assume that the rule is valid in my system, and this is supported by the high degree of linearity in plots as in Figures 3-1 and 3-2. A deviation from the rule of the geometric mean would result in a non-linear dependence of the relaxation data on the atom fraction of deuterium in the solvent.

The value of the fractionation factor for the water ligands of cobalt in $Co(H_2O)_6^{2+}$ was dependent on whether it was determined from T_1 or T_2, while that of $Ni(H_2O)_6^{2+}$ was independent of T_1 or T_2 (Table 3-2). These fractionation factors are a weighted value of all the protons that experience the paramagnetic relaxation. An explanation for this difference in ϕ_{T_1} and ϕ_{T_2} is based on the analysis of the contributions to the relaxation at 300 MHz (see Chapter 2). The paramagnetic contribution to the transverse relaxation of water protons in solutions of $Co(H_2O)_6^{2+}$ has a predominant contribution of water in the inner

Table 3-4: Calculated fractionation factors for the metal-bound water in the complexes of cobalt with glycine(gly), *N,N*-dimethylglycine (DMG), and acetylacetone (acac).

	ϕ_{T_1}	ϕ_{T_2}
$Co(H_2O)_6^{2+}$ [a]	0.95 ± 0.01	0.73 ± 0.02
$\phi Co(H_2O)_4(gly)^+$	0.91 ± 0.02	0.61 ± 0.03
$\phi Co(H_2O)_2(gly)_2$	0.79 ± 0.04	0.36 ± 0.03
$\phi Co(H_2O)_4(DMG)^+$	0.92 ± 0.02	0.62 ± 0.03
$\phi Co(H_2O)_2(DMG)_2$	0.84 ± 0.08	0.38 ± 0.05
$\phi Co(H_2O)_4(acac)^+$	0.96 ± 0.04	0.65 ± 0.04
$\phi Co(H_2O)_2(acac)_2$	1.04 ± 0.12	0.47 ± 0.07

Note: Calculated from equation 3-7 using the isotope effects on the formation constants of the reaction of Co^{2+} with glycine, *N,N*-dimethylglycine, and acetylacetone (Table 3-3) and ϕ_{T_1} and ϕ_{T_2} measured directly for $Co(H_2O)_6^{2+}$. A further description is in the text.

[a] Determined directly from NMR relaxation measurements.

coordination shell, suggesting that ϕ_{T_2} is measuring more of the fractionation of inner shell water. Since $T_{1\rho}$ has a larger contribution of outer shell water, ϕ_{T_1} is more of the fractionation of outer shell water. The analysis of the relaxation for $Ni(H_2O)_6^{2+}$ (Chapter 2) suggests that both T_1 and T_2 have a large contribution of outer shell water, so ϕ_{T_1} and ϕ_{T_2} for $Ni(H_2O)_6^{2+}$ each represent a larger contribution of the fractionation factor of outer shell water, and the influence of the fractionation of the inner shell water is not measured by ϕ_{T_1} or ϕ_{T_2}.

This interpretation of the fractionation factor for $Co(H_2O)_6^{2+}$ is consistent with expectations for the fractionation factor of metal-bound water. The fractionation factor for H_3O^+ is 0.69 (Chiang *et al.*, 1980), presumably due to the positive charge on the oxygen. It has been suggested that a metal-bound water site ought to have a fractionation factor approximated by $(0.69)^{\delta}$, with δ measuring the degree of positive charge transferred from the metal to the oxygen (Schowen and Schowen, 1982). The fractionation factor from T_2 for $Co(H_2O)_6^{2+}$ being 0.73 supports this suggestion in resembling the fractionation factor for H_3O^+. Pure water has by definition a fractionation factor of 1.0. Outer shell water, being much more loosely bound than inner shell water, should be much more like bulk water in its hydrogen / deuterium fractionation properties (More O'Ferrall *et al.*, 1971). The observation of ϕ_{T_1} for $Co(H_2O)_6^{2+}$ and ϕ_{T_1} and ϕ_{T_2} for $Ni(H_2O)_6^{2+}$ all being close to unity is consistent with these fractionation factors having a large contribution of the fractionation of outer shell water. The fractionation factor of the inner shell water in $Ni(H_2O)_6^{2+}$ may well be affected by the positive charge of the nickel and have a value closer to 0.69, or it may have a value near unity; however, because of the large contribution of outer shell water to both T_1 and T_2 in solutions of $Ni(H_2O)_6^{2+}$, the influence of the inner shell water is not large in the fractionation factors determined from T_1 and T_2.

The usefulness of fractionation factors is judged by their ability to aid in interpretation of solvent hydrogen isotope effects. I have determined the solvent hydrogen isotope effects on the formation of some simple inorganic complexes of Co^{2+} with the goal to use the knowledge of the fractionation of $Co(H_2O)_6^{2+}$ to interpret those isotope effects. An isotope effect is a function of all the fractionation factors for each hydrogenic site in a reaction (equation 1-2), and for a reaction such as equation 3-2 the fractionation factor of $Co(H_2O)_6^{2+}$ represents the individual contribution of those hydrogenic sites to the overall isotope effect on the reaction.

In considering the solvent hydrogen isotope effect on the formation constants (Table 3-3), initially I will assume that the isotope effect is made up entirely of the contribution of the isotope preferences of the hydrogenic positions of water in the inner coordination shell of $Co(H_2O)_6^{2+}$, $Co(H_2O)_4L^{+}$, and $Co(H_2O)_2L_2$. With this assumption there is enough information from the isotope effect and the fractionation factor of $Co(H_2O)_6^{2+}$ to calculate (from equation 3-7) the fractionation factor of the remaining waters in the cobalt-bidentate ligand complexes (Table 3-4). While I have done the calculations with equation 3-7 using both ϕ_{T_1} and ϕ_{T_2} for $Co(H_2O)_6^{2+}$ (Table 3-2), the values from ϕ_{T_1} may not be meaningful since T_1 is measuring more outer shell water, and the number of outer shell waters is not known. One interpretation of these calculations is that since ϕ_{T_2} of $Co(H_2O)_6^{2+}$ measures more of the fractionation of inner shell water in $Co(H_2O)_6^{2+}$, the calculation using ϕ_{T_2} of $Co(H_2O)_6^{2+}$ represents the resulting fractionation factor of the inner shell waters in the cobalt complexes. As ligands are added to Co^{2+} the calculated fractionation factor of the remaining coordinated waters decreases, and this decrease is the same for each of the three ligands, though glycine and *N,N*-dimethylglycine coordinate through an amino and carboxylate, and acetylacetone binds through two

carbonyls. If the assumption in these calculations is valid, the results of a lower fractionation factor for $Co(H_2O)_4L^+$ means that the O-H bond becomes weaker relative to that bond in $Co(H_2O)_6^{2+}$, and the O-H bond is also weaker in $Co(H_2O)_2L_2$ compared to $Co(H_2O)_4L^+$. This is similar to the effect of added ligands on the lifetime of water molecules in the hydration shell which, for instance, is shorter in $Co(H_2O)_4gly^+$ relative to $Co(H_2O)_6^{2+}$ (Hammes and Steinfeld, 1962), meaning that the hydration shell becomes more loosely bound when ligands are added. Thus in these cobalt complexes with bidentate ligands the metal-oxygen bond of a coordinated water is looser, and the oxygen hydrogen bond appears looser, relative to $Co(H_2O)_6^{2+}$.

However, from the measured solvent hydrogen isotope effects (Table 3-3) and the calculations of Table 3-4, there are a number of reasons to question the assumption that the only contribution to the isotope effects comes from the hydrogens of inner shell water. First, such strong fractionation as 0.36 (Table 3-4) is rare (Schowen and Schowen, 1982). Second, the lower fractionation factor for $Co(H_2O)_4L^+$ and $Co(H_2O)_2L_2$ is in contrast to the suggestion that the charge on the cobalt affects the fractionation of the metal-bound water (Schowen and Schowen, 1982). In these complexes, the net charge on the complex is +1 for $Co(H_2O)_4L^+$, and neutral for $Co(H_2O)_2L_2$, since the ligand has a charge of -1 in each case. Thus one would expect the fractionation factor of the waters in the ligand complexes to be closer to unity, according to the expectation that $\phi = (0.69)^{\delta}$, where 0.69 is the fractionation factor of H_3O^+ and δ is the degree of positive charge transferred from the metal to the oxygen of the coordinated water molecule (Schowen and Schowen, 1982). The ϕ_{T_2} for $Co(H_2O)_6^{2+}$ is consistent with this (Table 3-2), and thus one would expect [ϕ for $Co(H_2O)_6^{2+}$] < [ϕ for $Co(H_2O)_4L^+$] < [ϕ for $Co(H_2O)_2L_2$], but this opposite to what was observed (Table 3-4). Finally, based upon the large number of hydrogenic

sites in $Co(H_2O)_6^{2+}$, large isotope effects would be expected if the only contributions to the isotope effect came from the fractionation of inner shell water (Kresge *et al.*, 1987). However, isotope effects of unity were observed for these reactions (Table 3-3).

I suggest that the individual contribution of the fractionation factor of the inner shell waters in $Co(H_2O)_6^{2+}$, $Co(H_2O)_4L^+$, and $Co(H_2O)_2L_2$ to the overall solvent hydrogen isotope effect on the formation constants is small. The fractionation factor of some other hydrogenic sites has a contribution to the isotope effect which is sufficient to cancel the contribution of the inner shell waters so that the overall isotope effect is unity. The most likely choice for this is the fractionation of outer shell water. Other possibilities include, in the case of glycine, the composite fractionation factor of the hydration shell of the carboxylate group, but this would be a small contribution of a fractionation factor value already close to unity (the fractionation factor of the hydration shell of the carboxylate group in the acetate ion is 0.89, Goodall and Long, 1968). The fractionation factor of the hydrogens on the nitrogen in glycine are probably not important since the isotope effect is the same for *N,N*-dimethylglycine (Table 3-3).

The importance of outer shell water in ligand substitution reactions is not without precedent. The first step in the proposed mechanism of ligand substitution reactions is the formation of an outer shell complex with the ligand and metal (Eigen and Tamm, 1962), followed by replacement by the ligand of a water molecule in the inner coordination shell of the metal. Hence it is reasonable to assume that the binding of a ligand would affect the structure of outer shell water.

The following example illustrates how the fractionation of outer shell water could contribute to the isotope effect. Equation 3-7 can be expanded to

include the fractionation factor of outer shell water of $Co(H_2O)_6^{2+}$ in the numerator and of $Co(H_2O)_4L^+$ in the denominator. The number of outer shell waters would be larger than the number of inner shell waters, and if the contribution of an outer shell hydrogenic site in equation 3-7 was 1.06, twelve outer shell waters (24 hydrogenic positions) would contribute $(1.06)^{24} = 4.05$. This would then mean that the fractionation factor of the inner shell waters in $Co(H_2O)_4L^+$ is 0.74. If the contribution of an outer shell hydrogenic site is 1.10, then the fractionation factor of the inner shell waters in $Co(H_2O)_4L^+$ is 0.83. Thus a contribution of outer shell water slightly larger than unity would, when raised to a large power for the number of hydrogenic positions, cancel the effect of the inner shell waters.

I conclude that the solvent hydrogen isotope effect of unity on the formation of complexes of Co^{2+} and glycine, *N,N*-dimethylglycine, acetylacetone points to the importance of outer shell water in these reactions. Conversely, one can not get information about the effect of added ligands on the fractionation properties of the inner shell waters from the solvent hydrogen isotope effect on the formation constants. This work shows that the solvent hydrogen isotope effect is the complex contribution of the hydrogen / deuterium fractionation of several different groups or species of water.

CHAPTER 4
HYDROGEN / DEUTERIUM FRACTIONATION FACTOR OF THE AQUEOUS LIGAND OF COBALT IN Co(II)-SUBSTITUTED CARBONIC ANHYDRASE

Introduction

Carbonic anhydrase is a zinc metalloenzyme which catalyzes the reversible hydration of carbon dioxide to produce bicarbonate and a proton.

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$$

The aqueous ligand of the metal at the active site of carbonic anhydrase is believed to have a role in the catalytic pathway. Direct nucleophilic attack by the metal-bound hydroxide on CO_2 is a likely step in the production of bicarbonate (Pocker and Sarkanen, 1978; Lindskog, 1983; Silverman and Lindskog, 1988), and an intramolecular proton transfer involving the metal-bound water is proposed to be a rate-limiting step in catalysis by isozyme II (Steiner *et al.*, 1975). The solvent hydrogen isotope effect associated with the turnover number for CO_2 hydration catalyzed by native zinc-carbonic anhydrase II is 3.8 (Steiner *et al.*, 1975). To interpret fully the solvent hydrogen isotope effects observed for catalysis by carbonic anhydrase requires knowledge of the fractionation factor of the aqueous ligand of the metal.

The fractionation factor measures the tendency of deuterium to accumulate at the aqueous ligand of the metal relative to the deuterium content of bulk solvent. There are few reports of the fractionation factor of the aqueous ligands of a metal at the active site of an enzyme. Using NMR methods, Silverman (1981) has measured such a factor for the water ligands of cobalt in cobalt(II)-substituted carbonic anhydrase II ($\phi = 1.05 \pm 0.17$) by taking

advantage of the properties of a paramagnetic metal to enhance the relaxation rate of water exchanging rapidly between bulk solvent and the hydration shell of the metal and measuring the proton relaxation rate as a function of the deuterium content of solvent. Silverman measured that fractionation factor using T_1 at a proton resonance frequency of 100 MHz. Because of the unique relaxation properties of water protons in solutions of $Co(H_2O)_6^{2+}$ at 300 MHz (Chapter 2), I have determined the fractionation factor of the aqueous ligand of cobalt in three mammalian isozymes of Co(II)-substituted carbonic anhydrase from T_1 and T_2 at 300 MHz. In each case $T_1 > T_2$, implying that the same relaxation mechanisms occur for water protons in solutions of Co(II)-substituted carbonic anhydrase at 300 MHz as in solutions of $Co(H_2O)_6^{2+}$. The values of the fractionation factor determined from T_1 for each isozyme of Co(II)-substituted carbonic anhydrase were close to the fractionation factor for bulk water, which is unity. Except in the case of Co(II)-substituted carbonic anhydrase III, the fractionation factors obtained from T_2 were less than unity and close to the fractionation factor for H_3O^+ which is 0.69. I conclude that fractionation factors in these cases determined from T_1 and T_2 measured isotope preferences for different populations of ligand sites. I suggest that since T_2 has a large contribution from a paramagnetic chemical shift, the fractionation factors determined from T_2 have a large contribution of the hydrogen / deuterium fractionation of the inner shell ligand. The fractionation factors determined from T_1 are close to unity, the value in bulk water, and contain a larger contribution of the fractionation of outer shell water.

Experimental Procedure

Enzymes: Human carbonic anhydrase I was purified from human red blood cells by an affinity chromatography method (Kalifah *et al.,* 1977). Bovine carbonic anhydrase II was obtained from Sigma Chemical and used after

extensive dialysis to remove paramagnetic impurities. Bovine carbonic anhydrase III was obtained from bovine flank steak by gel filtration and anion-exchange chromatography (Tu *et al.*, 1986). The concentration of carbonic anhydrase I, II, and III was estimated at 280 nm using $\varepsilon = 4.7 \times 10^4\ M^{-1}\ cm^{-1}$ (Nyman and Lindskog, 1964), $5.4 \times 10^4\ M^{-1}\ cm^{-1}$ (Nyman and Lindskog, 1964), and $6.4 \times 10^4\ M^{-1}\ cm^{-1}$ (Engberg and Lindskog, 1984), respectively.

The apoenzymes of carbonic anhydrase I and II were prepared according to the procedure of Hunt *et al.* (1977) by dialysis against dipicolinic acid. Co(II) substituted carbonic anhydrase I and II were prepared by the addition of 1.1 equivalents of $CoCl_2$ followed by dialysis against several changes of large volumes of deionized water. The apoenzyme of carbonic anhydrase III was prepared by addition of the chelator 2-carboxy-1,10-phenanthroline to a solution of enzyme followed by dialysis against excess $CoCl_2$ as described by Engberg and Lindskog (1984).

NMR Measurements: Measurements at 300 MHz were the same as in Chapter 2. In the cases of Co(II)-substituted carbonic anhydrase I and II, $1/T_{io}$ was taken as the relaxation of solutions of the Co(II)-substituted enzyme in the presence of a molar excess of the inhibitor acetazolamide, which is known to displace the aqueous ligand of the metal at the active site. Enough acetazolamide was present to bind to 99.9% of the active sites. Alternatively for Co(II)-isozymes I and II and solely for Co(II)-substituted carbonic anhydrase III, $1/T_{io}$ was taken as the relaxation of solutions of the native zinc enzyme, which has no paramagnetic contribution. These two methods were equivalent at a frequency of 300 MHz for Co(II)-substituted carbonic anhydrase I and II. Solutions of enzyme for relaxation measurements were buffered with 50 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] at pH 8.5. At this pH the relaxivity of solutions of Co(II)-substituted carbonic anhydrase I and II is

greatest and in a region that is independent of pH (Wells *et al.*, 1979). (See Chapter 6 for a discussion of the pH and magnetic field dependence of the relaxation of water protons in solutions of Co(II)-substituted carbonic anhydrase III.) The values of pH reported here are uncorrected pH meter readings. The correction of a pH meter reading in 100% D_2O (pD = meter reading + 0.4) is approximately offset by the change in ionization state of the buffer in D_2O (pK_{D_2O} - pK_{H_2O} = 0.5 ± 0.1 for almost all acids with pK values between 3 and 10, Schowen, 1978). Water used for solution preparations was distilled and passed through two ion-exchange resin cartridges (Cole-Palmer 1506-35). D_2O (99.8%) was stirred with activated charcoal, the charcoal filtered out, and then the D_2O was distilled.

Measurement of the Hydrogen / Deuterium Fractionation Factor: The hydrogen / deuterium fractionation factor was determined by measuring the relaxation of water protons in solutions of Co(II)-substituted carbonic anhydrase as a function of the atom fraction of deuterium in solvent water as described in Chapter 3.

Results

The substitution of cobalt for zinc at the active site of carbonic anhydrase provides a paramagnetic center which affects the relaxation of water protons in solutions of carbonic anhydrase. The paramagnetic relaxivity of these water protons is greater for R_2 than for R_1 for each isozyme of carbonic anhydrase (Table 4-1).

The relaxation of water protons in solutions of the three isozymes of Co(II)-substituted carbonic anhydrase as a function of the atom fraction of deuterium in solvent water is shown in Figures 4-1, 4-2, and 4-3. The hydrogen / deuterium fractionation factors calculated from equation 3-4 using T_1 were near unity for the three isozymes (Table 4-2). However, the fractionation factors

Table 4-1: Paramagnetic contribution to the relaxivities of water protons observed for aqueous solutions of Co(II)-substituted carbonic anhydrase I, II, and III.

	frequency (MHz)	R_1 ($mM^{-1}s^{-1}$)	R_2 ($mM^{-1}s^{-1}$)	T_{1p}/T_{2p}
Co(II)-carbonic anhydrase I	300	0.28	2.3	8.2
Co(II)-carbonic anhydrase II	300	0.26	1.4	5.3
Co(II)-carbonic anhydrase III	300	0.14	2.2	16

Note: Measurements were made at 23 °C for solutions of enzyme buffered at pH 8.5 by 50 mM Hepes. Solutions contained 20% D_2O by volume.

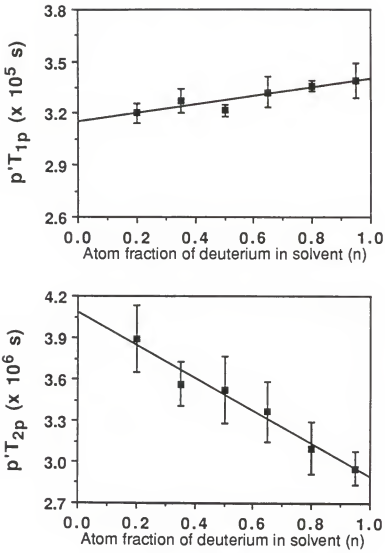


Figure 4-1: The dependence of $p'T_{ip}$ (i=1,2) at 300 MHz on **n**, the atom fraction of deuterium in solvent water, for 1.5 mM solutions of Co(II)-substituted carbonic anhydrase I. Solutions contained 50 mM Hepes buffer at pH 8.5 and 23°C. Data are the means and standard errors of three measurements.

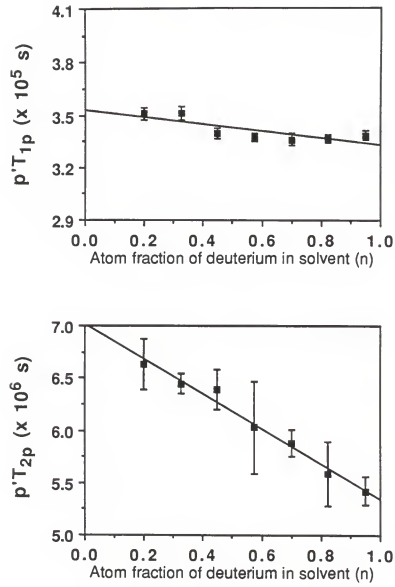


Figure 4-2: The dependence of $p'T_{ip}$ (i=1,2) at 300 MHz on **n**, the atom fraction of deuterium in solvent water, for 2.0 mM solutions of Co(II)-substituted carbonic anhydrase II. Solutions contained 50 mM Hepes buffer at pH 8.5 and 23°C. Data are the means and standard errors of three measurements.

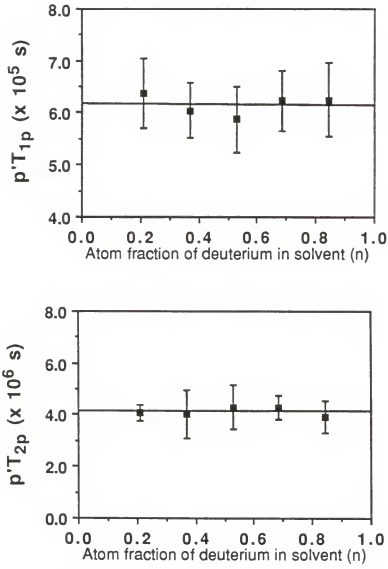


Figure 4-3: The dependence of $p'T_{ip}$ (i=1,2) at 300 MHz on **n**, the atom fraction of deuterium in solvent water, for 1.5 mM solutions of Co(II)-substituted carbonic anhydrase III. Solutions contained 50 mM Hepes buffer at pH 8.5 and 23°C. Data are the means and standard errors of three measurements.

Table 4-2: Hydrogen/deuterium fractionation factors determined from T_1 and T_2 at 300 MHz for the aqueous ligand of the paramagnetic metal in three mammalian isozymes of Co(II)-substituted carbonic anhydrase.

	ϕ_{T_1}	ϕ_{T_2}
Co(II)-carbonic anhydrase I	1.10±.02	0.72±.02
Co(II)-carbonic anhydrase II	0.95±.02	0.77±.01
Co(II)-carbonic anhydrase III	1.03±.06	1.00±.07

Note: Data were obtained from the least squares slope and intercept and standard error in a plot such as Figure 4-1 according to equation 3-4. Measurements were made at 23 °C for solutions of enzyme buffered at pH 8.5 by 50 mM Hepes.

calculated using T_2 were significantly less than unity for isozymes I and II, but near unity for isozyme III (Table 4-2).

Discussion

The relaxation behavior of water protons at 300 MHz and pH 8.5 in solutions of the Co(II)-substituted carbonic anhydrases showed T_{1p}/T_{2p} much greater than unity (Table 4-1). This was the same as observed for the water protons in solutions of cobalt ions (see Chapter 2), and the interpretation in the case of $Co(H_2O)_6^{2+}$ can be applied to Co(II)-substituted carbonic anhydrase. At 300 MHz, there is a field dependent increase in the chemical shift difference between a proton in the metal-bound site and the free solvent site. This causes an increase in the transverse relaxation of the protons as they experience the two environments (equation 2-3). Because this relaxation mechanism has as its source a paramagnetic contact shift, the transverse relaxation has a larger contribution of the ligand in the inner coordination shell of the cobalt, while the longitudinal relaxation has a larger contribution of water in the outer coordination shell. Thus the fractionation factor measured from T_2 represents more of the fractionation of the inner shell aqueous ligand of cobalt in Co(II)-substituted carbonic anhydrase, while the fractionation factor measured from T_1 is a greater contribution of the fractionation of outer shell water. A similar situation in which the outer shell relaxation has a larger contribution to R_1 than to R_2 has been reported by Kushnir and Navon (1984) for water protons in solution with Mn(II)-substituted carbonic anhydrase II.

These relaxation measurements were made at pH 8.5, a pH for which the relaxation rate of water protons in solutions of Co(II)-substituted carbonic anhydrase I and II is greatest (Wells *et al.*, 1979). (See Chapter 6 for an investigation of this property in solutions of Co(II)-substituted carbonic anhydrase III.) This pH is above the value of the pK for the ionization of the

active site group as determined in many experiments (see Table 5-1 and Engberg and Lindskog, 1984), especially spectrophotometric titrations and activity measurements, and the ligand of cobalt is believed to be a hydroxide ion at this pH. This presents some uncertainty as to which protons are actually being relaxed at pH 8.5 since a tetracoordinate cobalt-bound hydroxide ion would not be expected to exchange rapidly with water in solution. This issue has been discussed by Koenig *et al.* (1983) who proposed that ligand exchange with solvent involved a pentacoordinate intermediate having both OH^- and H_2O as ligands. Proton exchange can be rapid between these two ligands so that the departing H_2O can contain the oxygen of the initially-bound OH^-. On the basis of ^{18}O-exchange kinetics, Tu and Silverman (1985) suggested that the NMR relaxation observed at pH 8.5 is that of a water molecule hydrogen bonded to the cobalt-bound hydroxide yet close enough to the metal to be strongly relaxed. Yet another possibility is the rapid exchange of the hydrogen of the metal-bound hydroxide without exchange of the oxygen. Because of these unresolved issues, I cannot state specifically which of the hydrogens near the metal are the main contributors to the observed fractionation factors.

The fractionation factors measured from T_1 for each isozyme of Co(II)-substituted carbonic anhydrase were close to unity (Table 4-2), like ϕ_{T_1} for $Co(H_2O)_6^{2+}$ (Table 3-2), consistent with these values having a large contribution from the fractionation of outer shell water. This is reasonable from distance considerations, as it can be calculated using a $1/r^6$ dependence that the paramagnetic contribution to the longitudinal relaxation rate for a cobalt-bound hydroxide with a cobalt-proton distance of 2.8 Å is about the same as the paramagnetic contribution of the protons of three water molecules at an average distance of 3.8 Å. Also, it is known from crystallographic data that there

are a large number of water molecules within the active site cavity. Specifically, in human carbonic anhydrase II there are an estimated 9 water molecules between histidine 64 and the zinc, a distance of about 6 Å from the x-ray coordinates (Erickson *et al.,* 1986). The fractionation factors measured from T_2, ϕ_{T_2}, were near 0.72 for both Co(II)-substituted isozymes I and II, but 1.0 for isozyme III (Table 4-2), and reflect a larger contribution of the hydrogen / deuterium fractionation of the inner shell aqueous ligand. It is difficult to account for these differences, however, and though these isozymes have very similar amino acid sequences, they have unique active site and kinetic properties (Silverman and Vincent, 1983; Silverman and Lindskog, 1988). Isozyme II has the highest CO_2 hydration activity, with a turnover number of 10^6 s^{-1}. There is a histidine in position 64 which protrudes into the active site cavity of isozyme II. Isozyme I has a turnover number of 2×10^5 s^{-1}, and has His-64 as well as two other histidines, 67 and 200, in the active site cavity, and it is known that histidine 200 interacts strongly with the ligands of zinc (Kalifah, 1977). The active site cavity of isozyme I is smaller compared with that of isozyme II. Isozyme III has a turnover number of 10^4 s^{-1} and has a significant amount of positive charge in the active site, with a lysine in position 64 and an arginine in position 67. This positive charge may influence the ionization properties of the metal bound water in carbonic anhydrase III, for which the pK of the metal-bound water is less than 5.5, compared with isozyme I and II which have a pK around 7 (Tu *et al.,* 1983; Engberg and Lindskog, 1984). This difference in charge in the active site may also affect the hydrogen / deuterium fractionation properties of the ligand of the metal in Co(II)-substituted carbonic anhydrase III. Although isozymes I and II have very similar amino acid sequences, the significant differences in their structure, residues in the active site cleft, and catalytic activities (Pocker and Sarkanen, 1978; Lindskog, 1983; Silverman and

Lindskog, 1988) apparently do not have a significant effect on the fractionation factors which were nearly identical for isozymes I and II.

CHAPTER 5
INTERPRETATION OF SOLVENT HYDROGEN ISOTOPE EFFECTS ASSOCIATED WITH CARBONIC ANHYDRASE

Introduction

A solvent hydrogen isotope effect on a reaction is made up of individual contributions to the overall isotope effect from each hydrogenic site in the reaction (Schowen, 1978). This contribution is measured by the hydrogen / deuterium fractionation factor of each particular hydrogenic site. In considering reactions of carbonic anhydrase, the fractionation factor of the aqueous ligand of the metal represents a reactant state contribution that can be used to interpret equilibrium solvent hydrogen isotope effects which are a function of reactant and product state site fractionation factors, and kinetic isotope effects which are a function of reactant and transition state site fractionation factors.

Halide ions are inhibitors of carbonic anhydrase which bind to the metal and displace the water ligand of the metal (Bertini *et al.,* 1982). I have determined the solvent hydrogen isotope effect on the equilibrium binding of iodide ion to Co(II)-substituted carbonic anhydrase I and II and interpreted this effect using the fractionation factor of the aqueous ligand of cobalt in Co(II)-substituted carbonic anhydrase measured from NMR relaxation rates. The results suggest that the isotope effect on I^- binding binding is the complex contribution of the fractionation of more than one hydrogenic position, because the consideration of the fractionation factor of the aqueous ligand of cobalt by itself does not allow a complete interpretation of the isotope effect.

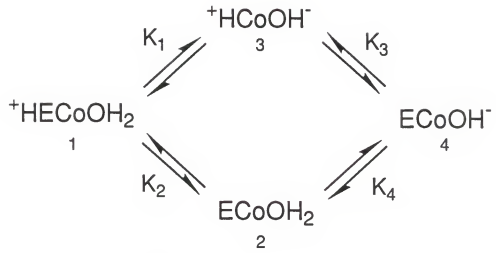
The fractionation factor of the aqueous ligand of cobalt in Co(II)-substituted carbonic anhydrase can also be used to interpret kinetic isotope

effects, and because a kinetic isotope effect includes the contribution of transition state hydrogenic sites, this allows a prediction of the transition state structure. In the case of Co(II)-substituted carbonic anhydrase II the fractionation factor measured from NMR leads to a predicted transition state structure that includes a proton transfer to a water molecule in the active site cavity. This is consistent with the large isotope effect on k_{cat} (Steiner *et al.,* 1975) and the nonlinear dependence of k_{cat} on the atom fraction of deuterium in solvent water (Venkatasubban and Silverman, 1980).

Experimental Procedure

The equilibrium dissociation constants describing the binding of iodide ion to Co(II)-substituted carbonic anhydrase were determined from visible absorption measurements (Lindskog, 1966). The pH dependence of the absorbance at 640 nm in solutions of Co(II)-substituted carbonic anhydrase I and II was measured on a Beckman DU-7 spectrophotometer. Stock solutions of enzyme at low pH contained 12.5 mM Hepes and 25 mM Mops (4-morpholinepropanesulfonic acid) buffer, and at high pH 0.5 M of triethylenediamine. The stock solutions had equal concentrations of enzyme. The titrations were performed by adding small increments of the high pH enzyme solution to the low pH enzyme solution. In this way the enzyme concentration stayed the same though the volume increased slightly. These titrations were done in H_2O and D_2O in the presence and absence of iodide ion which has been shown to bind to the metal site in carbonic anhydrase II (Brown *et al.,* 1977).

In the absence of anion, the data were fit (RS1, BBN Software, Cambridge, MA) to a scheme involving 4 micro equilibrium constants (Simonsson and Lindskog, 1982; Bertini *et al.,* 1985). In this scheme, only 3 of the 4 microconstants are independent, that is $K_1K_3 = K_2K_4$ (Scheme 5-1). When



Scheme 5-1: A diagram of two significant ionizations at and near the active site of Co(II)-substituted carbonic anhydrase II. One is the ionization of metal-bound water and the second is suggested to be the ionization of His-64 (Simonsson and Lindskog, 1982), the imidazole ring of which is about 6 Å from the metal. For Co(II)-substituted carbonic anhydrase I the second ionization could be either that of His-64 or His-200 (Whitney and Brandt, 1976).

anion was present, Scheme 5-1 was expanded to include the binding of the inhibitor, and it was assumed that the anion binds only to species 1 and 2 (see Tibell *et al.* (1984) for an estimate of the accuracy of this assumption). Thus two separate binding constants were determined. $K_1^{I^-}$ is the equilibrium dissociation constant of the anion complex with species 1 of Scheme 5-1, and $K_2^{I^-}$ is the equilibrium dissociation constant of the anion complex with species 2.

Results

In the absence of anion, the visible pH titration data fit Scheme 5-1 very well (Table 5-1). The constants K_1, K_2, and K_3 were determined from a fit to the data, and then K_4 was calculated from K_1, K_2, and K_3. The 4 microconstants determined in H_2O for Co(II)-substituted carbonic anhydrase II agreed very well with those obtained by Simonsson and Lindskog (1982), and the values determined in H_2O for Co(II)-substituted carbonic anhydrase I also agreed fairly well with those obtained by Bertini *et al.* (1985). In the presence of iodide the pH dependence of the absorbance at 640 nm was shifted to higher pH. Iodide binds with greater affinity to the diprotonated species 1 in Scheme 5-1 than to the monoprotonated species 2 (Table 5-2). This is similar to the iodide inhibition results of Simonsson and Lindskog (1982) for native zinc carbonic anhydrase II determined from activity measurements, and the results of Bertini *et al.* (1985) for nitrate inhibition of Co(II)-substituted carbonic anhydrase II determined from spectrophotometric titrations. The change in solvent from H_2O to D_2O has no measurable effect on $K_1^{I^-}$ and $K_2^{I^-}$ for Co(II)-substituted carbonic anhydrase I, but for Co(II)-substituted carbonic anhydrase II the change in solvent from H_2O to D_2O results in a decrease in these equilibrium constants (Table 5-2).

Table 5-1: Solvent hydrogen isotope effects on the acid-base microequilibrium constants observed for Co(II)-substituted carbonic anhydrase I and II.

	Co(II)-carbonic anhydrase I		Co(II)-carbonic anhydrase II	
	value in H_2O	ΔpK^a	value in H_2O	ΔpK^a
pK_1	7.55 ± 0.05	0.54 ± 0.06	5.65 ± 0.04	0.42 ± 0.05
pK_2	7.58 ± 0.15	0.45 ± 0.19	5.73 ± 0.07	0.37 ± 0.10
pK_3	8.11 ± 0.15	0.45 ± 0.19	6.95 ± 0.08	0.38 ± 0.12
pK_4	8.09 ± 0.22	0.54 ± 0.28	6.87 ± 0.12	0.43 ± 0.17

Note: The microconstants were determined from the variation of the absorbance at 640 nm with pH at 23°C and fit to Scheme 5-1. The solutions were buffered with Mops, Hepes and triethylenediamine with no other added ions as described in the experimental procedure.

[a] $\Delta pK = (pK_i)_{D_2O} - (pK_i)_{H_2O}$

Table 5-2: Solvent hydrogen isotope effects on the equilibrium dissociation constants of iodide ion with Co(II)-substituted carbonic anhydrase I and II.

	Co(II)-carbonic anhydrase I		Co(II)-carbonic anhydrase II	
	value in H_2O	ΔpK^{I-} [a]	value in H_2O	ΔpK^{I-} [a]
pK_1^{I-}	2.95 ± 0.10	-0.09 ± 0.17	3.48 ± 0.13	0.47 ± 0.19
pK_2^{I-}	2.35 ± 0.06	-0.07 ± 0.11	2.79 ± 0.02	0.27 ± 0.02

Note: The dissociation constants were determined from the pH dependence of the absorbance at 640 nm in the presence of 10 mM I^- with conditions as in Table 5-1. K_1^{I-} is the dissociation constant of I^- binding to species 1 of Scheme 5-1, and K_2^{I-} is the dissociation constant of I^- binding to species 2 of Scheme 5-1.

[a] $\Delta pK = (pK_i^{I-})_{D_2O} - (pK_i^{I-})_{H_2O}$

Discussion

I have discussed in Chapter 4 the fractionation factor for the exchangeable hydrogen at the active site of Co(II)-substituted carbonic anhydrase in its high pH form. The first application of the fractionation factors determined in Chapter 4 is to calculate the fractionation factor of the active site in its low pH form. Since the paramagnetic contribution to the relaxivity decreases to a very small value below pH 6.0 (Wells *et al.*, 1979), except at very low ionic strength (Bertini *et al.*, 1978; Bertini *et al.*, 1980), the fractionation factor cannot be measured directly from the relaxation rates at low pH. Instead, this was calculated from the fractionation factor of the high pH form and the solvent hydrogen isotope effect on the ionization constant of the active site, metal-bound water.

The visible pH titration data of isozymes I and II indicate that more than one ionization at or near the active site governs the absorbance at 640 nm (Bertini *et al.*, 1980). Previous investigators (Simonsson and Lindskog, 1982; Bertini *et al.*, 1985) have attributed this pH dependence to the ionization of two groups, the metal-bound water and another group near the active site which may be His-64 for isozyme II and probably His-64 or His-200 in isozyme I (Kalifah, 1977; Whitney and Brandt, 1976). I have measured the pH dependence of the absorbance at 640 nm for isozymes I and II in H_2O and D_2O and fit the data according to Scheme 5-1 to determine the solvent hydrogen isotope effect on the microequilibrium constants (Table 5-1). In Scheme 5-1, K_1 and K_4 are the constants for the metal-bound water, and K_2 and K_3 are the constants for the other active site group. Since the solvent hydrogen isotope effects on these equilibrium constants are all similar, within experimental uncertainty, it appears that the protonation state of one of the groups does not affect the hydrogen / deuterium fractionation properties of the other (consistent

with the conclusion that active site properties do not seem to influence the fractionation factors of the aqueous ligand of cobalt in Co(II)-substituted carbonic anhydrase I and II, see Chapter 4). Then the calculation to determine the fractionation factor of the water ligand of the metal can be performed neglecting the ionization state of the second ionizable group.

The isotope effect on an equilibrium constant is the product of all of the reactant state fractionation factors divided by the product of all of the product state fractionation factors (equation 1-2). For this ionization at the active site of Co(II)-substituted carbonic anhydrase

$$\geq Co{\cdot}OH_2 + H_2O \rightleftharpoons \geq Co{\cdot}OH + H_3O^+$$

the solvent hydrogen isotope effect can be written

$$\frac{K_{H_2O}}{K_{D_2O}} = \frac{(\phi_{Co\text{-}OH_2})^2(\phi_{H_2O})^2}{(\phi_{Co\text{-}OH})(\phi_{H_3O^+})^3}$$

From this measured isotope effect, the measured fractionation factor for the cobalt-bound hydroxide, the fractionation factor for H_3O^+ which has been measured to be 0.69 (Chiang, 1980), and the fractionation factor for H_2O which is 1.0, the fractionation factor for the exchangeable hydrogens at the active site in its low pH form can be calculated and are presented in Table 5-3. I have done the calculations of Table 5-3 using both ϕ_{T_1} and ϕ_{T_2} measured for the high pH form of the aqueous ligand of cobalt in Co(II)-substituted carbonic anhydrase I and II. However, the calculation from T_1 may not be meaningful, since it contains a large contribution of outer shell water. The value calculated using ϕ_{T_2} represents more closely the fractionation factor value of the inner shell cobalt-bound water.

Table 5-3: Values of the hydrogen / deuterium fractionation factor for the low pH forms of Co(II)-substituted carbonic anhydrase.

	ϕ_{T_1}	ϕ_{T_2}
Co(II)-substituted carbonic anhydrase I	1.12 ± 0.05	0.90 ± 0.04
Co(II)-substituted carbonic anhydrase II	0.90 ± 0.03	0.81 ± 0.03

Note: The ϕ for the low pH form was calculated using the isotope effect on the ionization constant of the cobalt-bound water and the ϕ for the high pH form measured directly from NMR and given in Table 4-2.

To apply the fractionation factors to an equilibrium solvent hydrogen isotope effect associated with the enzyme, I have studied at equilibrium the binding of I^- to Co(II)-substituted carbonic anhydrase I and II. I^- is known to bind to the metal in Co(II)-substituted carbonic anhydrase I and II displacing the coordinated water molecule (Brown *et al.*, 1977). Also, I^- binds fairly tightly (Table 5-2) and its size is comparable to that of a water molecule. Thus the isotope effect on the binding of I^- to Co(II)-substituted carbonic anhydrase may be simple enough to be accounted for by the fractionation factor of the metal-bound water.

The solvent hydrogen isotope effect on the binding of the iodide ion to the active site of carbonic anhydrase is given by

$$\geq Co\text{-}I \quad + H_2O \rightleftharpoons \geq Co\text{-}OH_2 + I^-$$

$$\frac{(K_i^{I^-})_{H_2O}}{(K_i^{I^-})_{D_2O}} = \frac{(\phi_{H_2O})^2}{(\phi_{Co\text{-}OH_2})^2}$$

where $(K_i^{I^-})_{H_2O}$ is the equilibrium dissociation constant describing the binding of I^- to species i of Scheme 5-1 in H_2O. Because it is known that $\phi_{H_2O} = 1.0$, with knowledge of $\phi_{Co\text{-}OH_2}$ it should be possible to estimate an isotope effect. Using ϕ_{T_2} for $\phi_{Co\text{-}OH_2}$ from Table 5-3, I thus predict that $\Delta pK^{I^-} = 0.09 \pm 0.04$ for Co(II)-isozyme I, and 0.18 ± 0.03 for Co(II)-isozyme II. The predicted value for isozyme I is in rough agreement with the measured value (Table 5-2), and the predicted value for isozyme II is slightly below the measured value. However, the predicted value for isozyme I is less than the predicted value for isozyme II, which is the same as in the experimental case. I have considered other interactions which may be taken into account for I^- binding. One of these is the composite fractionation factor of the solvation shell of I^- ($\phi = 0.59$, Voice, 1974).

Including this in the denominator of the above equation for the isotope effect increases the predicted value, which is then closer to the experimental value for Co(II)-isozyme II, but farther from the experimental value for Co(II)-isozyme I. This implies that I have incomplete knowledge of the fractionation properties of each hydrogenic site that is affected when iodide ion binds to the enzyme. However, to include the fractionation factors of any other groups in the equation requires much speculation about the fractionation factors near the active site. Thus it is likely that the isotope effect must be described by the complex contribution of the fractionation factors of additional groups or water associated with the enzyme, and that the fractionation factor of the aqueous ligand of the metal is by itself not sufficient to account for the isotope effect on I^- binding.

The discussion of the solvent hydrogen isotope effect on iodide binding suggests that the fractionation factor can be used to interpret at least qualitatively isotope effects associated with carbonic anhydrase. It is also possible to apply the fractionation factors for the aqueous ligand of the metal in Co(II)-substituted carbonic anhydrase in the interpretation of isotope effects on kinetic constants associated with the enzyme to see if similar information can be obtained. A kinetic isotope effect is the product of the reactant state fractionation factors divided by the product of the transition state fractionation factors (equation 1-1). The isotope effect on k_{cat} for the CO_2 hydration activity of Co(II)-substituted carbonic anhydrase II is 1.8 (R. S. Rowlett, unpublished), and the rate limiting step measured by k_{cat} is the intramolecular transfer of a proton from the metal-bound water to regenerate the metal-bound hydroxide for the next round of catalysis (Steiner *et al.*, 1975). This proton transfer from the metal-bound water is thought to go through a water-bridge to histidine-64 in the active site cleft, from which the proton can then be released to solvent

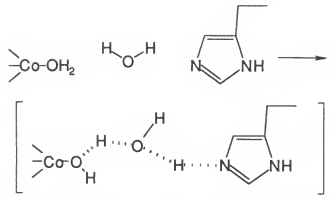
(Venkatasubban and Silverman, 1980). For this individual step in the catalysis, the reaction is

$$Co\text{-}OH_2 + H_2O + His \longrightarrow Co\text{-}OH + H_2O + His\text{-}H^+$$

and the isotope effect is

$$\frac{k_{H_2O}}{k_{D_2O}} = \frac{(\phi_{Co\text{-}OH_2})^2(\phi_{H_2O})^2}{\prod_{i}^{\text{all transition state sites}} \phi_i^T}$$

The hydrogenic sites of water have a fractionation factor of 1.0, and the hydrogenic sites of the cobalt-bound water have a fractionation factor of 0.81 (Table 5-3). Using these values and the isotope effect in the above equation, the product of the transition state fractionation factors can be calculated to be 0.36. In the transition state, the proton remaining on the metal-bound oxygen is in transition between a metal bound water which has a fractionation factor of 0.81 (Table 5-3), and a metal-bound hydroxide which has a fractionation factor of 0.77 (Table 4-2), so I will arbitrarily use a value of 0.79 as the fractionation factor of this hydrogenic site in the transition state. Therefore, the product of the fractionation factors of the other hydrogenic sites in the transition state is 0.36/0.79 = 0.46. These other hydrogenic sites in the transition state are the positions associated with the water bridge between the metal-bound water and the histidine in the active site cleft.



This calculation for the fractionation factor of the water bridge in the transition state suggests that the protons of the water bridge have a fractionation factor less than unity, meaning they have hydronium ion character, consistent with positive charge transferred to the oxygen during the proton transfer process. This calculation of the fractionation factor of the transition state suggests that the proton transferred from the cobalt-bound water is in between the cobalt-bound water and the water bridge in the transition state. As in the case of iodide binding to Co(II)-substituted carbonic anhydrase, there may be other hydrogenic sites in the transition state which contribute to the overall isotope effect. However, the fractionation factors of the aqueous ligand of the metal have led to this qualitative model of the transition state, which is consistent with both the large isotope effect on k_{cat} and the results of Venkatasubban and Silverman (1980) for the catalysis of CO_2 hydration in H_2O / D_2O mixtures.

CHAPTER 6
MAGNETIC FIELD AND pH DEPENDENCE OF THE WATER PROTON NMR RELAXATION ENHANCEMENT IN SOLUTIONS OF Co(II)-SUBSTITUTED CARBONIC ANHYDRASE III

Introduction

Carbonic anhydrase III is a mammalian isozyme of carbonic anhydrase found primarily in skeletal muscle. It is a less efficient catalyst of CO_2 hydration compared with isozymes I and II, which are found in red blood cells (Lindskog, 1983). Another distinctive feature of carbonic anhydrase III is the pH independence of its catalytic activity in the range pH 6-9, in which carbonic anhydrase II has an inflection at pH 7 and maximal activity at high pH (Tu *et al.*, 1983; Kararli and Silverman, 1985; Tu *et al.*, 1986). It is also apparent that the zinc is bound more tightly in carbonic anhydrase III than in isozymes I and II (Engberg and Lindskog, 1984).

The replacement of cobalt for zinc in carbonic anhydrase I and II has proved useful to study the properties of the metal at the active site (Bertini and Luchinat, 1983). The catalytic and visible spectral properties of Co(II)-substituted carbonic anhydrase III have been investigated (Engberg and Lindskog, 1984). I report here the NMR relaxation enhancement of water protons in solutions of Co(II)-substituted carbonic anhydrase III. I found, in contrast to Co(II)-substituted carbonic anhydrase I and II, no pH dependence of the NMR relaxation in the range of pH 6-9. Also, the frequency dependence of the paramagnetic contribution to the relaxation shows an increase with higher frequency, as opposed to the dispersion to lower relaxation rates observed in solutions of Co(II)-substituted carbonic anhydrase I and II.

Experimental Procedure

The purification of bovine carbonic anhydrase III and preparation of Co(II)-substituted carbonic anhydrase III was performed as described in Chapter 4. The total concentration of carbonic anhydrase III was estimated at 280 nm by using $\varepsilon = 6.4 \times 10^4\ M^{-1}\ cm^{-1}$ (Engberg and Lindskog, 1984). The concentration of Co(II)-substituted carbonic anhydrase III was estimated at 640 nm by using the extinction coefficients determined by Engberg and Lindskog (1984). Zinc analysis of the native enzyme yielded 1.03 ± 0.05 zinc per molecule of enzyme. Cobalt analysis of the Co(II)-substituted enzyme yielded 0.33 ± 0.04 cobalt per molecule of enzyme, with the remainder apoenzyme. The fraction of the sample that was apoenzyme was restored to full activity by the addition of zinc.

The longitudinal relaxation rates of the protons of solvent water in solutions of Co(II)-substituted and native Zn-carbonic anhydrase III were measured using a field cycling "relaxometer" (Koenig *et al.*, 1983) at the IBM T. J. Watson Research Center in Yorktown Heights, New York. The rates were measured at 5°C at pH 6.0 (50 mM Mes/NaOH buffer), pH 7.0 (50 mM Mops/NaOH buffer), pH 8.0 (50 mM Hepes/NaOH buffer), and pH 9.0 (50 mM Ches/NaOH buffer) in solutions containing 1 mM of enzyme. The pH 6 samples were also measured at 25°C. The samples were in equilibrium with atmospheric CO_2. The total relaxivity, R_t, is defined as in equation 6-1

$$R_t = (1/T_1 - 1/T_{1o})/N \qquad (6\text{-}1)$$

where $1/T_1$ is the observed relaxation rate of the protein solution, $1/T_{1o}$ is the relaxation rate of the buffer, and N is the millimolar concentration of protein. At each pH the relaxivity was determined as a function of frequency over the range

0.01 to 50 MHz. The paramagnetic relaxivity, R_p, is R_t determined for a solution of Co(II)-substituted carbonic anhydrase III minus R_t for a solution of native Zn-carbonic anhydrase III.

Results

The frequency dependence of the total relaxivity was similar to the NMR dispersion profiles of other native and Co(II)-substituted carbonic anhydrases (Fabry *et al.*, 1970; Wells *et al.*, 1979) (Figure 6-1). The total relaxivity is greatest at low frequency, then begins to decrease at about 0.1 MHz. The paramagnetic relaxivity, however, showed a small value at low frequency, then at about 0.5 MHz R_p began to increase to a maximal value at about 5 MHz, and then began to decrease again (Figure 6-2). The total relaxivities for both the Co(II)-substituted and native Zn-carbonic anhydrase III showed a possible increase with pH at 1 MHz, but remained constant with pH at 40 MHz (Figure 6-3). This may be the same effect reported by Wells *et al.* (1979), who observed that the diamagnetic component of the relaxivity in solutions of Co(II)-substituted and native zinc carbonic anhydrase II increases with pH at low frequency. When only the paramagnetic relaxivity was considered (the difference between R_t of Co(II)-substituted carbonic anhydrase III and R_t of native zinc carbonic anhydrase III shown in Figure 6-3), there was no pH dependence, especially when the data at pH 7 in Figure 6-3 are excluded.

Discussion

This work is the first study of NMR relaxation enhancement of water protons in solutions of Co(II)-substituted carbonic anhydrase III. Previous work with isozyme III has shown that the visible spectrum of Co(II)-substituted carbonic anhydrase III is very similar to the high pH spectral forms of Co(II)-substituted carbonic anhydrase I and II; however, for Co(II)-substituted carbonic anhydrase III there is no pH dependence of the spectrum in the range pH 6-9

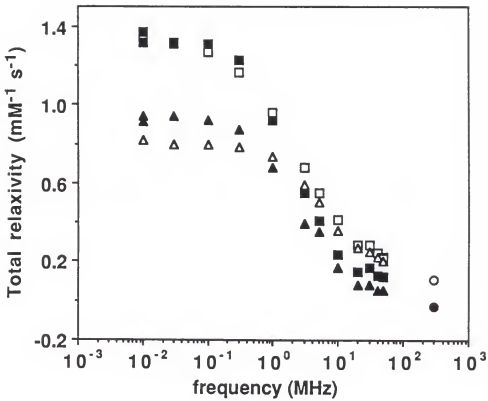


Figure 6-1: The total relaxivity of Co(II)-substituted carbonic anhydrase III and native zinc carbonic anhydrase III as a function of the proton resonance frequency. The solutions contained 1 mM enzyme at pH 6.0 (50 mM Mes / NaOH), pH 8.0 (50 mM Hepes / NaOH), or pH 9.0 (50 mM Ches / NaOH). pH 6.0 Co(II)-substituted carbonic anhydrase III at 5°C (△), pH 6.0 native zinc carbonic anhydrase III at 5°C (▲), pH 9.0 Co(II)-substituted carbonic anhydrase III at 5°C (□), pH 9.0 native zinc carbonic anhydrase III at 5°C (■), pH 8.0 Co(II)-substituted carbonic anhydrase III at 23°C (○), pH 8.0 native zinc carbonic anhydrase III at 23°C (●).

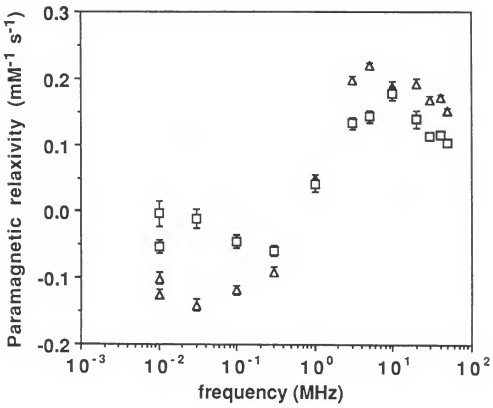


Figure 6-2: The paramagnetic relaxivity of Co(II)-substituted carbonic anhydrase III as a function of the proton resonance frequency. Experimental conditions as in Figure 6-1. pH 6.0 (Δ), pH 9.0 (□).

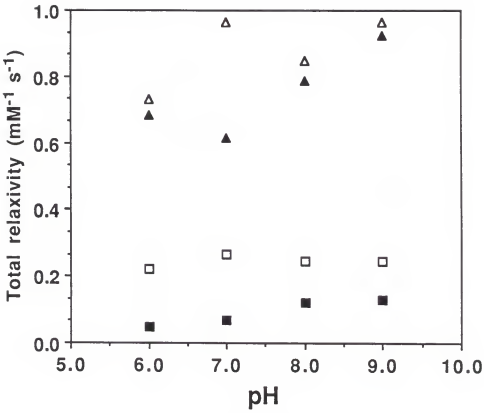


Figure 6-3: The total relaxivity of Co(II)-substituted carbonic anhydrase III and native zinc carbonic anhydrase III as a function of pH. Experimental conditions as in Figure 6-1. Co(II)-substituted carbonic anhydrase III measured at 1 MHz (△), native zinc carbonic anhydrase III measured at 1 MHz (▲), Co(II)-substituted carbonic anhydrase III measured at 40 MHz (□), native zinc carbonic anhydrase III measured at 40 MHz (■).

(Engberg and Lindskog, 1984). Also, the CO_2 hydration activity of the cat isozyme III and the bovine isozyme III is independent in the pH range 6-8.5 (Tu *et al.*, 1983; Engberg *et al.*, 1985). These data imply that the activity controlling group in Co(II)-substituted and native Zn-carbonic anhydrase III, the aqueous ligand of the metal, is predominantly in the hydroxide form in the pH range 6-9, and that the pK for the ionization of the metal-bound water to the metal-bound hydroxide is well below 6.

NMR relaxation enhancement studies with Co(II)-substituted carbonic anhydrase I and II have shown a large pH dependence of the paramagnetic relaxation rate in the pH range 6-9, with an inflection at pH 7 and maximal relaxation at high pH (Fabry *et al.*, 1970; Wells *et al.*, 1979). This correlates with the pH dependence of both the visible spectrum and CO_2 hydration activity (Bertini *et al.*, 1982; Silverman and Vincent, 1983). Thus the NMR relaxation enhancement seems to correlate with the ionization state of the aqueous ligand of the metal in Co(II)-substituted carbonic anhydrase I and II.

However, it has been shown that SO_4^{2-} present in solution to maintain ionic strength affects the pH dependence of both the visible spectrum and the paramagnetic relaxation of Co(II)-substituted carbonic anhydrase I and II (Bertini *et al.*, 1978; Bertini *et al.*,1980; Simonsson and Lindskog, 1982). When SO_4^{2-} and other anions are absent in either unbuffered solutions or solutions containing only sulfonic acid buffers, the visible spectrums of Co(II)-substituted carbonic anhydrase I and II still show a large pH dependence, but there is practically no pH dependence on the paramagnetic contribution to the longitudinal relaxation rate of water protons in solutions Co(II)-substituted carbonic anhydrase II (Bertini *et al.*, 1978), and a small dependence for Co(II)-substituted carbonic anhydrase I (Bertini *et al.*, 1980). The data in Figure 6-3 are from solutions containing no anions other than those of the sulfonic acid

buffer. Under these conditions, the relaxation of water protons in solutions of Co(II)-substituted carbonic anhydrase II would be independent of pH in the range 6-9, and this is what was observed for the paramagnetic relaxivity of solutions of Co(II)-substituted carbonic anhydrase III (Figure 6-3).

The reason for the small value of R_p at low frequency (Figure 6-2) is not clear. One possibility is that the lifetime of a proton in the hydration shell of the cobalt is too long to allow for a large paramagnetic contribution (Fabry *et al.*, 1970; Tu *et al.*, 1985). This water off rate has a maximal value of 10^4 s^{-1} in the case of native carbonic anhydrase III (Tu *et al.*, 1983), but is 10^5 s^{-1} for Co(II)-substituted carbonic anhydrase II (Tu *et al.*, 1985). This rate would be expected to be even slower at 5°C, but when the pH 6 sample was measured at 25 °C, there was a larger paramagnetic relaxivity at low frequency (data not shown). Another possibility is that in this isozyme the cobalt is in a pentacoordinate or hexacoordinate environment (Engberg and Lindskog, 1984), which would have less ability to relax due to a shorter electron relaxation time (Koenig *et al.*, 1983). Whatever the reason, the paramagnetic contribution at low frequency is small.

The increase in R_p with higher frequency (Figure 2) is unlike that for any other Co(II)-substituted carbonic anhydrase which show a decrease in R_p with higher frequency (Wells *et al.*, 1979), but is not unlike that observed for Mn, Cu, and Ni proteins (Koenig and Brown, 1973; Bertini and Luchinat, 1986). While the relaxation behavior of these systems is not completely understood, it is clear that the field dependence of the electron spin relaxation is a factor. Thus, the increase in the electron relaxation time may be responsible for the increase in R_p at intermediate frequency. The decrease at higher frequency may be due to the $\omega_s\tau_c$ dispersion predicted by the Solomon-Bloembergen equations (Bertini and Luchinat, 1986).

CHAPTER 7
CONCLUSIONS

This work shows the utility of the NMR relaxation method to measure the hydrogen / deuterium fractionation properties of the aqueous ligands of cobalt in some specific cases. In particular, the characterization of the relaxation mechanisms at 300 MHz has allowed the detection of the fractionation of hydrogen and deuterium of two populations of ligand sites associated with cobalt, one having a significant contribution of the inner shell aqueous ligand of the metal and the other apparently dominated by outer shell water. The fractionation factor for the inner shell ligand can differ significantly from that for solvent water, and in the case of $Co(H_2O)_6^{2+}$, ϕ_{T_2} is near the fractionation factor of H_3O^+, implying that the water ligands of cobalt in $Co(H_2O)_6^{2+}$ are influenced by the positive charge of the cobalt. The fractionation factors of Co(II)-substituted carbonic anhydrase represent the first direct measurement of this parameter at the active site of an enzyme.

The conclusions of the contributions of inner and outer shell water to the relaxation times T_1 and T_2 result from the effect of the chemical shift relaxation mechanism at 300 MHz. This conclusion is supported by calculations and experimental evidence with EDTA for $Co(H_2O)_6^{2+}$. In this hexaaquacomplex the only interactions are those of the metal and water, and the water can be in the inner shell metal-bound site, an outer shell site, or the bulk solvent site. There are no other effects of the medium in this system. In the case of Co(II)-substituted carbonic anhydrase, there are many other interactions which may occur to affect the fractionation of the hydrogenic positions of the aqueous

ligand of the metal. These interactions include influences of charge by residues near the active site, and the effects of the hydrogen bond network that exists in the active site cavity. This hydrogen bond network includes water in the active site cavity and specific residues from the enzyme, as well as the aqueous ligand of the metal. Thus it is clear that outer shell water in Co(II)-substituted carbonic anhydrase would have some different properties than outer shell water in $Co(H_2O)_6^{2+}$. The relaxation rates at 300 MHz of water protons in solution of Co(II)-substituted carbonic anhydrase showed T_1 much larger than T_2, as in $Co(H_2O)_6^{2+}$, and this indicates that the chemical shift mechanism affects T_2 in solutions of Co(II)-substituted carbonic anhydrase. However there is no other support for this from calculations or other experiments, as in the case of $Co(H_2O)_6^{2+}$, except that the fractionation factor determined from T_1 and T_2 are also different for Co(II)-substituted carbonic anhydrase. This inference of the chemical shift mechanism affecting T_2 in solutions of Co(II)-substituted carbonic anhydrase is the only extrapolation made from $Co(H_2O)_6^{2+}$ to Co(II)-substituted carbonic anhydrase, which are very different systems in many other respects. It is important to note that the fractionation factors for Co(II)-substituted carbonic anhydrase are measured directly, and thus are independent of any other interpretation regarding $Co(H_2O)_6^{2+}$, except that of the contribution of inner shell water to T_2 and of outer shell water to T_1. Thus the fractionation factors for Co(II)-substituted carbonic anhydrase include the effects of the interactions near the active site.

One very unique opportunity to understand the basis for the differences in the fractionation factors between the isozymes of carbonic anhydrase exists in the study of site-directed mutants of the enzyme. It is known that the pK for the ionization of the metal-bound water in carbonic anhydrase III is lower than that ionization in carbonic anhydrase I and II. This difference is most likely due

to the positively charged residues near the active site in carbonic anhydrase III. The fractionation factor for the metal-bound hydroxide in carbonic anhydrase III was greater than that fractionation factor in carbonic anhydrase I and II, and this may also be due to the positively charged residues in carbonic anhydrase III. This could be tested, for instance, by replacing the histidine in position-64 of carbonic anhydrase I and II with a lysine, which occupies position-64 in carbonic anhydrase III, and then measuring the fractionation factor using the cobalt-substituted enzyme. One would predict that the fractionation factor of Co(II)-substituted carbonic anhydrase I and II with lysine in position-64 would be closer to unity, like that in Co(II)-substituted carbonic anhydrase III.

The value of the fractionation factor for $Co(H_2O)_6^{2+}$ measured directly from NMR represents a reactant state fractionation factor that can be used to interpret solvent hydrogen isotope effects associated with $Co(H_2O)_6^{2+}$. The solvent hydrogen isotope effects on the formation of complexes of cobalt with some bidentate ligands suggest that the fractionation factor of some other hydrogenic positions must contribute to the overall isotope effect, most likely the positions of outer shell water in these complexes, because the isotope effect could not be accounted for by considering only the fractionation factor of $Co(H_2O)_6^{2+}$. This may be because of the effect of the bidentate ligands on the structure of outer shell water in these complexes. It could be that the fractionation factor of the outer shell water in a cobalt-bidentate ligand complex is different than that fractionation factor in $Co(H_2O)_6^{2+}$. Also, it may be that the number of outer shell waters that experience the paramagnetic relaxation enhancement by the cobalt is less in the cobalt-bidentate ligand complex than in $Co(H_2O)_6^{2+}$. However, it is clear that there would be a large number of outer shell waters associated with complexes, and that a small effect on the fractionation of the outer shell water in these complexes on going from the

reactant to the product state, when raised to a large power for the number of hydrogenic positions in the outer shell water, could have a large contribution to the overall isotope effect.

The values of the fractionation factor of the aqueous ligand of the metal at the active site of Co(II)-substituted carbonic anhydrase can also be used to interpret solvent hydrogen isotope effects on reactions associated with carbonic anhydrase. The solvent hydrogen isotope effect on the equilibrium binding of iodide ion to Co(II)-substituted carbonic anhydrase suggests that there must be other hydrogenic positions whose isotope preference changes on going from the reactant to product states, because the consideration of the fractionation factor for the aqueous ligand of cobalt in Co(II)-substituted carbonic anhydrase by itself does not allow a complete interpretation of the isotope effect. However, it is clear from the results that the hydrogenic positions of the metal-bound water at the active site do make a large contribution to the overall isotope effect. This means that the other hydrogenic positions that contribute to the isotope effect probably have a fractionation factor just slightly different from unity, but the large number of these positions would then give a significant contribution to the overall isotope effect. The solvent hydrogen isotope effects for other anions binding to Co(II)-substituted carbonic anhydrase could be determined, and even for other inhibitors of the enzyme that are known to bind to the active site. This may give more information about these other hydrogenic positions. For instance, it may be that the fractionation factor of the hydration shell of the anion is important, and this could be determined from the results with other monovalent anions. Also, bulkier groups could be used, such as the sulfonamide inhibitors, and these may displace more outer shell water within the active site cavity. The isotope effect on the binding of a sulfonamide

inhibitor may then give some insight into the contribution of these outer shell hydrogenic positions to the overall isotope effect.

To account for the solvent hydrogen isotope effects on a rate constant is even more complex because knowledge of the fractionation factor values for the transition state are required. The model for the transition state of the rate-limiting proton transfer step of the CO_2 hydration reaction, developed from the fractionation factor of the aqueous ligand of the cobalt in Co(II)-substituted carbonic anhydrase II and the isotope effect on k_{cat}, is consistent with other experimental evidence for the structure of the transition state. Other kinetic isotope effects on reactions of carbonic anhydrase may then also be used with the fractionation factor to get information about the mechanisms of these reactions. For instance, the solvent hydrogen isotope effects on the binding of anions could be measured, and then interpreted with the fractionation factors of the reactant state. This may show whether the rate-limiting step of the binding is the initial entry of the anion into the active site cavity, or it may be that the final binding of the anion to the metal is the rate-limiting step. This work represents an important first step in the accurate interpretation of the solvent hydrogen isotope effects in the catalysis by carbonic anhydrase.

Perhaps the most significant achievement in this work is the development of the NMR relaxation method to measure directly the hydrogen / deuterium fractionation factor of the hydrogenic positions of water coordinated to metals. This method could also be used with other transition metals and in other types of inorganic complexes, and in other metalloenzymes. Also, the conclusions of the contributions of inner and outer shell water to the relaxation times T_1 and T_2 could also be studied further by, for instance, determining the effects of ionic strength and temperature and the relaxation and the fractionation factors

determined from T_1 and T_2. It is hoped that the work presented in this dissertation will be the catalyst for other studies in this field.

References

Baes, N. F.; Mesmer, R. E. *The Hydrolysis of Cations;* Wiley: New York, 1976.

Bernheim, R. A.; Brown, T. H.; Gutowsky, H. S.; Woessner, D. E. *J. Chem Phys.* **1959**, *30*, 950-956.

Bertini, I.; Canti, G.; Luchinat, C.; Scozzafava, A. *J. Am Chem Soc.* **1978**, *100*, 4873-4877.

Bertini, I.; Dei, A.; Luchinat, C.; Monnanni, R. *Inorg. Chem.* **1985**, *24*, 301-303.

Bertini, I.; Luchinat, C. *Acc. Chem. Res.* **1983**, *16*, 272-279.

Bertini, I.; Luchinat, C. *NMR of Paramagnetic Species in Biological Systems*; Benjamin-Cummings: Menlo Park, CA, 1986.

Bertini, I.; Luchinat, C.; Scozzafava, A.; *Inorg. Chem. Acta* **1980**, *46*, 85-89.

Bertini, I.; Luchinat, C.; Scozzafava, A. *Struct. Bonding (Berlin)* **1982**, *48*, 45-92.

Bloembergen, N. *J. Chem Phys.* **1957**, *27*, 572-573.

Bloembergen, N.; Morgan, L. O. *J. Chem. Phys.* **1961**, *34*, 842.

Brown, G. S.; Navon, G.; Shulman, R. G. *Proc. Natl. Acad. Sci. USA* **1977**, *74*, 1794-1797.

Carlson, G. A.; McReynolds, J. P.; Verhoek, F. H. *J. Am. Chem. Soc.* **1945**, *67*, 1334-1339.

Chiang, Y.; Kresge, A. J.; More O'Ferrall, R. A. *J. C. S. Perkin II* **1980**, 1832-1839.

Dwek, R. A. *NMR in Biochemistry;* Oxford press: Oxford, 1975.

Eigen, M.; Tamm, K. *Z. Elektrochem.* **1962**, *66*, 93-121.

Eisenstadt, M. *J. Chem Phys.* **1969**, *51*, 4421-4432.

Engberg, P.; Lindskog, S. *FEBS Lett.* **1984**, *170*, 326.

Engberg, P.; Millqvist, E.; Pohl, G.; Lindskog, S. *Arch. Biochem. Biophys.* **1985**, *241*, 628-638.

Eriksson, E. A.; Jones, T. A.; Liljas, A. In *Zinc Enzymes;* Bertini, I.; Luchinat, C.; Maret, W.; Zeppezauer, M. Eds.; Birkhauser: Boston, 1986; 317-328.

Fabry, M. E.; Koenig, S. H.; Schillinger, W. E. *J. Biol. Chem.* **1970**, *245*, 4256-4262.

Freeman, R.; Kempsell, S. P.; Levitt, J. *J. Magn. Reson.* **1980**, *138*, 453-479.

Friedman, H. L.; Holz, M.; Hertz, H. G. *J. Chem Phys.* **1979**, *70*, 3369.

Glasoe, P. K.; Long, P. A. *J. Phys. Chem.* **1960**, *64*, 188-191.

Gold, V.; Lowe, B. M. *J. Chem. Soc. (A)* **1968**, 1923-1932.

Gold, V.; Wood, D. L. *J. Chem. Soc., Dalton Trans.* **1982**, 2452-2461.

Goodall, D. M.; Long, F. A. *J. Am. Chem. Soc.* **1968**, *90*, 238.

Gueron, M. *J. Magn. Reson.* **1975**, *19*, 58.

Hammes, G. C.; Steinfeld, J. I. *J. Am. Chem. Soc.* **1962**, *84*, 4639-4643.

Hunt, J. B.; Rhee, M. J.; Storm, C. B. *Anal. Biochem.* **1977**, *79*, 614-617.

Jencks, W. P.; Salvesen, K. *J. Am Chem Soc.* **1971**, *93*, 4433.

Kalifah, R. *Biochemistry* **1977**, *16*, 2236-2240.

Kalifah, R, G.; Strader, D. J.; Bryant, S. H.; Gibson, S. M. *Biochemistry* **1977**, *16*, 2241-2247.

Kararli, T.; Silverman, D. N. *J. Biol. Chem.* **1985**, *260*, 3484-3489.

Koenig, S. H.; Brown, R. D. *Ann. N. Y. Acad. Sci.* **1973**, *222*, 752-763.

Koenig, S. H.; Brown, R. D.; Bertini, I.; Luchinat, C. *Biophys. J.* **1983**, *41*, 179-187.

Kresge, A. J.; More O'Ferrall, R. A.; Powell, M. F. In *Isotopes in Organic Chemistry,* Volume 7; Buncel, E.; Lee, C. Eds.; Elsevier: New York, 1987; 177-273.

Laughton, P. M.; Robertson, R. E. In *Solute-Solvent Interactions*; Coetzee, J. F.; Ritchie, C. D. Eds.; Marcel Dekker: New York, 1969; 399-538.

Lindskog, S. *Biochemistry* **1966**, *5*, 2641-2646.

Lindskog, S. in *Zinc Enzymes*; Spiro, T. G. Ed.; Wiley: New York; 1983; 78-121.

Lowe, B. M.; Smith, D. G. *J. Chem. Soc., Faraday Trans. I* **1975**, *71*, 389-397.

Luz, Z.; Meiboom, S. *J. Chem. Phys.* **1964**, *40*, 2686-2692.

Meiboom, S.; Gill, D. *Rev. Sci Instrum.* **1958**, *29*, 688.

Melamud, E.; Mildvan, A. S. *J. Biol. Chem.* **1975**, *250*, 8193-8201.

Melton, B. F.; Pollack, V. L. *J. Phys. Chem.* 1969, *73*, 3669-3679.

More O'Ferrall, R. A.; Koeppl, G. W.; Kresge, A. *J. Am. Chem. Soc.* **1971**, *93*, 9-20.

Morgan, L. O.; Nolle, A. W. *J. Chem Phys.* **1959**, *31*, 365.

Nyman, P. O.; Lindskog, S. *Biochim. Biophys. Acta* **1964**, *85*, 141-151.

Pocker, Y.; Sarkanen, S. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1978**, *47*, 149-274.

Schowen, K. B. J. In *Transition States in Biochemical Processes*; Grandour, R. D.; Schowen, R. L. Eds.; Plenum: New York, 1978; 225-284.

Schowen, K. B.; Schowen, R. L. In *Methods in Enzymology*, Volume 87; Purich, D. L. Ed.; Academic: New York, 1982; 551-606.

Silverman, D. N. *J. Am. Chem. Soc.* **1981**, *103*, 6242-6243.

Silverman, D. N.; Lindskog, S. *Acc. Chem. Res.* **1988**, *21*, 30-36.

Silverman, D. N.; Vincent, S. H. *CRC Crit. Rev. Biochem.* **1983**, *14*, 207-255.

Simonsson, I.; Lindskog, S. *Eur. J. Biochem.* **1982**, *123*, 29-36.

Smith, R. M.; Martell, A. E. *Critical Stability Constants;* Plenum: New York, 1975.

Solomon, I. *Phys. Rev.* **1955**, *99*, 559-565.

Steiner, H.; Jonsson, B.; Lindskog, S. *Eur. J. Biochem.* **1975**, *59*, 253-259.

Swift, T. J.; Connick, R. E. *J. Chem. Phys.* **1962**, *37*, 307-320; *J. Chem. Phys.* **1964**, *41*, 2553-2554.

Szawelski, R. J.; Wharton, C. W.; White, S. *Biochem. Soc. Trans.* **1982**, *10*, 232-233.

Tibell, L.; Forsman, C.; Simonsson, I.; Lindskog, S. *Biochim. Biophys. Acta* **1984**, *789*, 302-310.

Tu, C. K.; Sanyal, G.; Wynns, G. C.; Silverman, D. N. *J. Biol. Chem.* **1983**, *258*, 8867-8871.

Tu, C. K.; Silverman, D. N. *Biochemistry* **1985**, *24*, 5881-5887.

Tu, C. K.; Thomas, H. G.; Wynns, G. C.; Silverman, D. N. *J. Biol. Chem.* **1986**, *261*, 10100.

Venkatasubban, K. S.; Silverman, D. N. *Biochemistry* **1980**, *19*, 4984.

Voice, P.J. *J. C. S. Faraday I* **1974**, *70*, 498-505.

Wells, J. W.; Kandel, S. I.; Koenig, S. H. *Biochemistry* **1979**, *18*, 1989-1995.

Whitney, P. L.; Brandt, H. *J. Biol. Chem.* **1976**, *251*, 3862-3867.

BIOGRAPHICAL SKETCH

James W. Kassebaum, the son of Amanda L. Schoonmaker and Wilbur F. Kassebaum, was born March 6, 1962, in Mount Vernon, Illinois. James grew up in St. Louis, Missouri, and in 1980 graduated first in his class at Parkway South Senior High School. He then attended Purdue University, West Lafayette, Indiana, and in 1984 graduated with Honors with the degree of Bachelor of Science in chemistry. The summer of 1984 was a very eventful one, when James was wed to Shari L. Gunderman and then entered graduate school at the University of Florida in the Department of Biochemistry and Molecular Biology and studied under the supervision of Dr. David Silverman. James completed the requirements for the Ph.D. degree in 1988, and began his professional career at the Monsanto Company in St. Louis.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

David N. Silverman, Chair
Professor of Pharmacology and Therapeutics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

E. Raymond Andrew
Graduate Research Professor of Physics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Robert J. Cohen
Associate Professor of Biochemistry and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Russell S. Drago
Graduate Research Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Daniel L. Purich
Professor of Biochemistry and
Molecular Biology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1988

Dean, College of Medicine 10/25/88

Dean, Graduate School